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PRODUCTION OF GLUTATHIONE WITH MICROBIOLOGICAL ENERGY

KOUSAKU MURATA

1981

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MICROBIOLOGICAL ENERGY**

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ABBREVIATIONS

AMP : Adenosine 5'-monophosphate
ADP : Adenosine 5'-diphosphate
ATP : Adenosine 5'-triphosphate
AMN : Adenosine 5'-mononicotinate
ADPG: Adenosine 5'-diphosphateglucose
ADPR: Adenosine 5'-diphosphateribose
CMP : Cytidine 5'-monophosphate
CTP : Cytidine 5'-triphosphate
FBP : Fructose 1,6-bisphosphate
FAD : Flavine adenine dinucleotide
NAD : Nicotinamide adenine dinucleotide
NADP: Nicotinamide adenine dinucleotide phosphate
NTG : N-Methyl-N'-nitro-N-nitrosoguanidine
Tris: tris(hydroxymethyl)aminomethane

INTRODUCTION

Recent advances in the immobilization and stabilization of enzymes and microbial cells have greatly enhanced their potential for use as highly specific catalyst in large scale industrial processes. Immobilized enzymes and immobilized microbial cells have been utilized for the continuous production of biologically, medically and nutritionally important classes of compounds such as L-amino acids, L-malic acid, urocanic acid, 6-aminopenicillanic acid and so forth (Table 1)¹⁾. The applications of immobilized enzyme and immobilized cell techniques are now expanding rapidly in various fi-

elds such as organic synthesis, chemical and clinical analysis, food industry, medicine and so forth.

Thus, the enzyme and microbial cells have been used

economically and efficiently by immobilization, and the technique aimed at the immobilization has been the subject of increased interest in biotechnology since the end of the 1960s. However, up to now, industrial application of these enzymes has been limited almost entirely to the catalysis of degradation reactions and simple transformation reactions. The application of immobilized enzymes to the complicated synthetic processes has not been done.

Among the more exciting but as yet unexplored applications of enzymes will be those which involve synthesis of complicated

Table 1. Current applications of immobilized enzymes and microbial cells in industry

Enzymes and microbial cells	Applications	Operating since
Aminoacylase	Optical resolution of DL-amino acids	1969
Glucose isomerase	Isomerization of glucose to fructose	1973
Penicillin amidase	Production of 6-APA	1973
<i>Escherichia coli</i> (Aspartase)	Production of aspartate	1973
<i>Brevibacterium ammoniagenes</i> (Fumarase)	Production of L-malic acid	1974
β -Galactosidase	Hydrolysis of lactose	1977

molecules from the simple materials. In fact, many useful compounds, especially those produced by the conventional fermentative processes, are usually biosynthesized by multi-enzyme reactions in microbial cells. Therefore, the application of multi-step enzyme reactions to the production of useful compounds by the bioreactor system is an important problem and is now extensively studied in the world.

The barrier preventing the development of economically feasible processes of multi-step enzymatic synthesis may be the lack of an adequate (re)generation and/or recycle system for cofactors such as ATP, NAD, NADP, Coenzyme A and so on, which are essential for many biosynthetic reactions in microbial cells. Therefore, the construction of a (re)generation system of cofactors, especially ATP, is indispensable not only for the economic utilization of enzymes, but also for the process economy and reaction efficiency.

From this standpoint, Langer et al.²⁾ evaluated the relative merits of various routes to ATP (re)generation involving chemical synthesis, whole cells, organelles or sub-cellular systems and cell free enzymes (Table 2). However, all useful chemical synthesis of ATP involve nonaqueous media and usually require complicated long processes. The organelles such as mitochondria, chloroplasts and chromatophores also (re)generate ATP using photoenergy, but their ATP-(re)generating activities and useful life times are insufficient for the practical processes.

On the other hand, whole cells, disrupted cells and cell free enzymes are promising as ATP (re)generation systems, since these preparations show high ATP-(re)generating activities besides the high selectability of reactions. However, among these prepara-

tions, the utilization of whole microbial cells is more advantageous, since it excludes the intricate and tedious procedures for the preparation of disrupted cells or enzymes. Even if the whole microbial cells contain the undesirable reactions such as ATPase or phosphatases, such unwanted side reactions can be suppressed by appropriate physicochemical methods.

Thus, it is concluded that the utilization of whole micro-

Table 2. Comparison of phosphotransferases and subcellular organelles for ADP phosphorylation

Enzyme	Phosphate donor (BP)	Cost of BP	Stability of BP	Max. Equili. constant
Acetate kinase	Acetyl phosphate	High	Fair	400
Arginine kinase	L-Phosphoarginine	High	Poor	500
Aspartate kinase	4-Phospho-L-aspartate	High	-	2,800
Carbamate kinase	Carbamyl phosphate ¹	Moderate	Poor	25
Creatine kinase ²	Creatine phosphate	High	Good	100
Pyruvate kinase	Phosphoenolpyruvate	High	Good	6,600
Ammonia kinase ³	Phosphoramidate	Low	-	-
Polyphosphate kinase ⁴	Polyphosphate	Low	Good	-
3-Phosphoglycerate kinase	1,3-Diphospho-D-glycerate	High	-	3,400

1) By-products volatile

2) Mammalian enzyme source only

3) Labile enzyme, low cellular activity 4) Low cellular activity

Organelle	System	Stability
Mitochondria	Respiration chain	1-2 days (0 °C)
Chloroplasts	Cyclic phosphorylation	1-3 days (0 °C)
Chromatophores	Cyclic phosphorylation	1-3 days (0 °C)
(Glycolysis)	Glycolytic pathway	25 days (30 °C) ¹

1) Immobilized in polyacrylamide gel lattice

bial cells having an ATP-(re)generating activity has some merits for use in large scale commercial processes. Judging by the low cost of substrates and high activity and stability, the acetate kinase in bacterial cells and the glycolytic pathway in yeast

cells are promising as the enzyme or enzyme system catalyzing the (re)generation of ATP (Table 2).

The basis for the utilization of whole microbial cells as an ATP (re)generation system was first demonstrated by Tochikura and co-workers³⁾. They studied the utilization of the glycolytic pathway in yeast cells as an ATP (re)generation system and they succeeded in applying this system to the production of useful compounds such as cytidine diphosphate choline⁴⁾ and 6-phosphogluconic acid⁵⁾ by batch processes. From a industrial standpoint, however, a continuous reaction system using immobilized cells was considered to be more advantageous than a batch process, since by immobilization of cells the following advantages would be expected: (i) Stability of enzymes is improved, (ii) Enzymes can be reused, (iii) Continuous operation is possible, (iv) Reaction requires less space, (v) Control of reaction is easy, (vi) Highly pure products are obtained.

Thus, in order to develop a more efficient method, I studied the construction of an ATP (re)generation system using the acetate kinase reaction in *Escherichia coli* B or the glycolytic pathway in *Saccharomyces cerevisiae* cells. I also studied the application of this ATP (re)generation system to the bioreactor for the continuous production of useful compounds, especially glutathione.

Besides the development of an efficient bioreactor system, the improvement of the properties of microbial cells is also important problem. The construction of microbial cells having high productivity of useful compounds was also extensively studied by employing biochemical and gene engineering techniques.

This thesis consists of the following three main parts.

(i) demonstrating the utilization of energy in biomolecules (phos-

phate polymers) for the economical production of G-6-P and NADP. The properties of polyphosphate glucokinase and metaphosphate-dependent NAD kinase, an activity which I was the first to detect in microorganisms. (2) Continuous production of glutathione by immobilized microbial cells containing an ATP (re)generation system. In this section, I will describe the conditions for the construction of a highly efficient bioreactor for the production of glutathione. (3) Construction of *Escherichia coli* B strains having high glutathione-synthesizing activities by employing biochemical and gene engineering techniques. Finally, I will discuss the feasibilities of the ATP (re)generation systems for the industrial production of glutathione and other useful compounds.

CHAPTER I

Utilization of energy in phosphate polymers

Section 1 NADP production with phosphate polymers

1-1 Metaphosphate: A new phosphoryl donor for NAD phosphorylation

INTRODUCTION

Since the discovery of bathophilic substances in microorganisms by Jeener and Brachet⁶⁾, other investigators have described their occurrence in microorganisms, higher plants and animal tissues. This substance was initially thought to be a ribonucleic acid, but it was latter identified as a polymer of inorganic phosphates(polyphosphates) by Wiame⁷⁾. Since then, many investigations on the function of polyphosphate have been reported. For example, Stevenick and Booiij⁸⁾ indicated the important role of polyphosphate in active transport of glucose. Nishi^{9,10)} reported the specific role of polyphosphate in cell division. Harold¹¹⁾ also showed the binding of polyphosphate to the cell wall of *Neurospora crassa*. However, the most important finding which provided the basis for an explanation of the function of polyphosphate was demonstrated by Yoshida and Yamataka¹²⁾ and by Kornberg et al.^{13,14)}. They showed the existence of polyphosphate kinase(E.C. 2.7.4.1) which catalyzes the reversible transfer of phosphate between polyphosphate and ATP in *Escherichia coli*. Yoshida¹⁵⁾ also showed that the polyphosphate is a high energy compounds thermodynamically equivalent to ATP. These findings by Yoshida and Yamataka and by Kornberg and his colleagues indicated that the polyphosphate may serve as a primary phosphoryl donor in biological phosphorylating reactions.

In fact, many enzymes using metaphosphate, a ring phosphate

polymer, for phosphorylation of certain compounds were detected in some microorganisms. Szymona et al.^{16,17)} described a glucose phosphorylating enzyme that uses metaphosphate in an acetone powder of *Mycobacterium phlei* and they called this enzyme polyphosphate glucokinase (E.C. 2.7.1.a). They also detected a fructose phosphorylating enzyme in the same microorganism and called it polyphosphate fructokinase¹⁸⁾. Dirheimer and Ebel¹⁹⁾ found AMP phosphorylating enzyme that uses metaphosphate in *Corynebacterium xerosis*. Thus it seems certain that polyphosphate is utilized as a high energy compound for phosphorylation of biological molecules. But other explanations for the function of polyphosphate have been suggested. Of these explanations, a typical one is that the polyphosphate is only a reserve form of phosphate. Therefore, further studies on polyphosphate are now required to explain its basic functions.

Until now, only two NAD phosphorylating enzymes have been known in microorganisms and mammalian tissues. One of them is ATP-dependent NAD kinase (EC 2.7.1.23) first reported by Euler and Adler²⁰⁾ in yeast in 1938 and subsequently by Mehler et al.²¹⁾ in pigeon liver extracts. Since then, the activity of ATP-dependent NAD kinase has been detected in a wide variety of animal and plant tissues. Recently, another NAD phosphorylating activity was detected by Ogata et al.^{22,23)} in some microorganisms. They found that this was the reverse reaction of acid phosphatase. Therefore, the present enzyme utilizing metaphosphate for NAD phosphorylation is probably the third NAD phosphorylating enzyme and is different from the ATP-dependent NAD kinase and acid phosphatase. I distinguished this enzyme from others and tentatively called it metaphosphate-dependent NAD kinase.

In this section, I describe the distribution of metaphosphate-dependent NAD kinase in microorganisms. I also compare the properties of metaphosphate-dependent NAD kinase with ATP-dependent NAD kinase.

MATERIALS AND METHODS

Culture conditions All microorganisms were cultivated in Medium A or B. Medium A contained 1.0 % glucose, 1.0 % yeast extract, 1.0 % peptone, 0.5 % meat extract, 0.1 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5 % KH_2PO_4 (pH 7.0). Medium B contained 2.0 % glucose, 0.1 % $(\text{NH}_4)_2\text{SO}_4$, 0.01 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 M K_2HPO_4 and 0.05 % yeast extract removed inorganic phosphates by magnesia treatment²⁴. The culture was reciprocated at 30 °C for 20 h with 100 ml of medium in 500 ml Sakaguchi flasks.

Preparation of cell extracts Cells harvested by centrifugation were suspended in 5.0 mM Tris-HCl buffer (pH 7.0) and treated with a sonic oscillator (Kubota Model 200 M) for 5 min at 9 KHz. After centrifugation at 27,000 xg for 30 min, the supernatant was dialyzed against the same buffer overnight at 4 °C. The cell extracts thus obtained were used as enzyme sources.

Reaction system NAD phosphorylating reaction was carried out at 37 °C for 1-2 h in 1.0 ml mixture containing 5.0 mM NAD, 5.0 mM MgCl_2 , 2.0 mg/ml metaphosphate or 5.0 mM ATP, 0.1 M Tris-HCl buffer (pH 7.0) and cell extract (1.0-4.0 mg/ml protein). The reaction was terminated by immersing the test tube in boiling water for 5 min, and NADP formed was determined by the enzymatic method²⁵. Under these reaction conditions, enzyme activity was proportional to reaction time and protein concentration, respectively. Protein was determined by the method of Lowry et al.²⁶. The specific activity was expressed as μmol of NADP formed per milligram of protein per hour. Paper chromatography and paper electrophoresis were also used to identify the phosphorylated compounds. The solvent systems for the chromatographies and electrophoresis are shown in the table legends.

Chemicals Tri- and tetrametaphosphate and tripolyphosphate were obtained from Sigma Chemical Co., St. Louis, MO. Metaphosphate and polyphosphate were obtained from Katayama Chemical Industries Co., Osaka, Japan. NAD and NADP were purchased from Oriental Yeast Co., Tokyo, Japan. ADP and ATP were purchased from Kojin Co., Tokyo, Japan.

RESULTS

NAD phosphorylation by cell extract from B. ammoniagenes

NAD phosphorylating reactions were performed under various conditions to ascertain the existence of metaphosphate-utilizing

enzyme activity in *B. ammonia-*
genes cell extract. As can be
 seen in Table 1, NAD phosphory-
 lation proceeded enzymatically
 using metaphosphate as a sole
 source of phosphoryl donor, and
 Mg^{2+} accelerated this phospho-
 rylation. The addition of small
 amounts of ADP to reaction mix-
 ture had no effect. This indicates that the polyphosphate kinase
 (EC 2.7.4.1) reaction, which regenerates ATP by phosphorylating
 ADP using certain phosphate polymers, was not involved in this

TABLE I. COMPONENT REQUIREMENTS FOR
 NAD PHOSPHORYLATION

Reaction system	Specific activity ^a
Complete ^b	0.074
NAD omitted	0
Metaphosphate omitted	0
MgCl ₂ omitted	0.009
0.005 mM ADP added	0.072
0.010 mM ADP added	0.073
Complete ^c	0

^a μ mol of NADP formed/mg of protein/h.

^b Complete system is described in MATERIALS
 AND METHODS.

^c Boiled cell extract was used.

TABLE II. BEHAVIOR OF NAD PHOSPHORYLATING PRODUCT ON PAPER
 CHROMATOGRAPHIES AND PAPER ELECTROPHORESIS

Compounds	R _f values			Mobilities ^a	
	Solvent system ^b			Solvent System ^c	
	I	II	III	I	II
NAD	0.33	0.24	0.32	+ 4.2	+10.1
Product	0.22	0.11	0.52	+12.1	+12.9
Authentic NADP	0.23	0.11	0.54	+12.2	+12.6

^a Plus sign indicates migration distance in centimeter to the anode from the origin.

^b I, Isobutyrate-0.5 N NH₄OH (5:3 v/v); II, 95% ethanol-1.0 M CH₃COONH₄ (7:3 v/v); III, saturated
 (NH₄)₂SO₄-0.1 M CH₃COONa-isopropanol (40:10:1 v/v).

^c I, 0.2 M acetate buffer (pH 3.5), 3.0 kV, 30 min, 60 min; II, 0.1 M borate buffer (pH 9.3), 3.0 kV, 30 min,
 45 min.

phosphorylation. Thus it was clear that metaphosphate was used as
 phosphoryl donor for NAD phosphorylation by the enzyme from *B. am-*
moniagenes.

NAD phosphorylating product was analyzed. On paper chromato-
 graphs and high-voltage paper electrophoresis, the product gave
 the same R_f values and mobilities as authentic NADP (Table II),
 and no fluorescent and ultraviolet absorbing spots were found other
 than NAD and the product. The product was almost completely and

stoichiometrically reduced by the coupled reaction with glucose-6-phosphate dehydrogenase or isocitrate dehydrogenase (data not shown). These results indicated that the product was NADP.

Distribution of metaphosphate-dependent NAD kinase activities

Table III shows the distribution patterns of metaphosphate-dependent NAD kinase activities among microorganisms grown on Medium A. Metaphosphate-dependent NAD kinase activity was found in some limited genera, such as *Acetobacter*, *Achromobacter*, *Brevibacterium*, *Corynebacterium* and *Micrococcus*, though ATP-dependent NAD kinase activity was found in all microorganisms tested.

Effect of culture conditions on metaphosphate-dependent and ATP-dependent NAD kinase activities

1. Effect of culture time

B. ammoniagenes cells were grown on Medium B, and activity changes in the two NAD kinases were examined periodically (Table IV). The activities of the two NAD kinases decreased with increasing culture time, and the activity ratios were nearly equal.

2. Effect of inorganic phosphate concentrations

B. ammoniagenes cells were grown on Medium B containing K_2HPO_4 at various concentrations. The two NAD kinase activities were not affected by inorganic phosphate concentrations in medium, and the activity ratios were also nearly equal (Table V).

TABLE III. DISTRIBUTION OF METAPHOSPHATE- AND ATP-DEPENDENT NAD KINASE ACTIVITIES IN MICROORGANISMS

Strain	Specific activity ^a	
	Meta-P ^b -dependent NAD kinase	ATP-dependent NAD kinase
<i>Acetobacter xylinum</i>	0.018	0.023
<i>Achromobacter butyri</i>	0.017	0.055
<i>Enterobacter aerogenes</i>	0	0.053
<i>Agrobacter tumefaciens</i>	0	0.034
<i>Alcaligenes faecalis</i>	0	0.052
<i>Bacillus subtilis</i>	0	0.044
<i>Bacillus natto</i>	0	0.073
<i>Brevibacterium ammoniagenes</i>	0.075	0.060
<i>Brevibacterium flavum</i>	0.039	0.108
<i>Corynebacterium sepeidonicum</i>	0.029	0.006
<i>Escherichia coli</i> (Strain B)	0	0.044
<i>Escherichia coli</i> (Strain K-12)	0	0.023
<i>Escherichia coli</i> (Crookes)	0	0.045
<i>Micrococcus flavus</i>	0.025	0.034
<i>Micrococcus lysodeikticus</i>	0.031	0.030
<i>Micrococcus glutamicum</i>	0.050	0.021
<i>Pseudomonas aeruginosa</i>	0	0.020
<i>Proteus mirabilis</i>	0	0.096
<i>Serratia marcescens</i>	0	0.033
<i>Sarcina aurantiaca</i>	0	0.012

^a μ mol of NADP formed/mg-protein/h

^b Meta-P, metaphosphate

All microorganisms were grown on Medium A and metaphosphate- and ATP-dependent NAD kinase activities were assayed as described in MATERIALS AND METHODS.

3. Effect of culture aeration

B. ammoniagenes cells were grown on Medium B with various volume of culture medium. Metaphosphate-dependent NAD kinase activity was constant, though the ATP-dependent NAD kinase activity decreased with the increasing culture volume (Table VI).

4. Effect of carbon sources

B. ammoniagenes cells were grown on Medium B containing

TABLE IV. EFFECT OF CULTURE TIME ON METAPHOSPHATE- AND ATP-DEPENDENT NAD KINASE ACTIVITIES

Culture time (h)	Growth (O.D. at 660 m μ)	Specific activity ^a		Activity ratio (Meta-P/ATP)
		Meta-P ^b -dependent NAD kinase	ATP-dependent NAD kinase	
16	0.54	0.076	0.072	1.05
20	1.12	0.072	0.065	1.11
40	1.47	0.061	0.060	1.02
56	1.38	0.050	0.045	1.11

^a μ mol of NADP formed/mg of protein/h.

^b Meta-P, metaphosphate.

B. ammoniagenes cells were grown on Medium B. After several hour culture, cells were harvested, and the two NAD kinase activities were assayed as described in MATERIALS AND METHODS.

TABLE V. EFFECT OF INORGANIC PHOSPHATE CONCENTRATIONS IN MEDIUM ON METAPHOSPHATE- AND ATP-DEPENDENT NAD KINASE ACTIVITIES

K ₂ HPO ₄ in medium (mM)	Growth (O.D. at 660 m μ)	Specific activity ^a		Activity ratio (Meta-P/ATP)
		Meta-P ^b -dependent NAD kinase	ATP-dependent NAD kinase	
3.0	0.8	0.069	0.055	1.25
8.0	1.1	0.069	0.054	1.28
20	1.2	0.072	0.058	1.24
50	1.4	0.076	0.063	1.21
100	1.1	0.074	0.063	1.17

^a μ mol of NADP formed/mg of protein/h.

^b Meta-P, metaphosphate.

B. ammoniagenes cells were grown on Medium B containing K₂HPO₄ at the various concentrations indicated in the table. After 20 hr culture, cells were harvested, and the two NAD kinase activities were assayed as described in MATERIALS AND METHODS.

TABLE VI. EFFECT OF CULTURE VOLUME ON METAPHOSPHATE- AND ATP-DEPENDENT NAD KINASE ACTIVITIES

Culture volume (ml)	Growth (O.D. at 600 m μ)	Specific activity ^a		Activity ratio (Meta-P/ATP)
		Meta-P ^b -dependent NAD kinase	ATP-dependent NAD kinase	
15	1.4	0.075	0.077	0.97
45	1.3	0.069	0.067	1.03
100	1.1	0.074	0.058	1.28
200	0.6	0.073	0.045	1.62

^a μ mol of NADP formed/mg of protein/h.

^b Meta-P, metaphosphate.

B. ammoniagenes cells were grown on Medium B with various medium volume. After 20 hr culture, cells were harvested, and the two NAD kinase activities were assayed as described in MATERIALS AND METHODS.

TABLE VII. EFFECT OF CARBON SOURCES ON METAPHOSPHATE- AND ATP-DEPENDENT NAD KINASE ACTIVITIES

Carbon source (0.5 %)	Growth (O.D. at 660 m μ)	Specific activity ^a		Activity ratio (Meta-P/ATP)
		Meta-P ^b -dependent NAD kinase	ATP-dependent NAD kinase	
Glucose	1.0	0.078	0.068	1.15
Glycerol	0.6	0.079	0.066	1.20
Citrate	0.5	0.076	0.220	0.35
Succinate	0.8	0.075	0.210	0.36

^a μ mol of NADP formed/mg of protein/h.

^b Meta-P, metaphosphate.

B. ammoniagenes cells were grown on Medium B containing various carbon sources. After 20 hr culture, cells were harvested, and the two NAD kinase activities were assayed as described in MATERIALS AND METHODS.

various carbon sources. Metaphosphate-dependent NAD kinase activity was constant regardless of the carbon sources used for growth. On the other hand, ATP-dependent NAD kinase activity varied remarkably (Table VII). A three fold increase in ATP-dependent NAD kinase activity was observed when the cells were grown on succinate or citrate, both members of the tricarboxylic acid cycle.

Enzymatic properties of metaphosphate- and ATP-dependent NAD kinases in B. ammoniagenes

1) pH effect

Figure 1 shows the pH-activity curves of the two NAD kinases. The pH optimum for activity was 6.0 and 5.0 for metaphosphate- and ATP-dependent NAD kinase, respectively.

2) Heat stability

Figure 2 shows the heat stabilities of the two NAD kinases. Metaphosphate-depen-

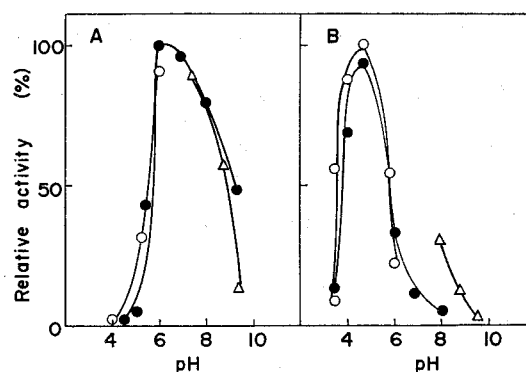


FIG. 1. Effect of pH on Metaphosphate (A)- and ATP (B)-Dependent NAD Kinase Activities in Extracts of *B. ammoniagenes*.

All buffers were used at the concentration of 0.1 M. Buffers were: ○, acetate buffer; △, Tris-HCl buffer; ●, Tris-maleate buffer.

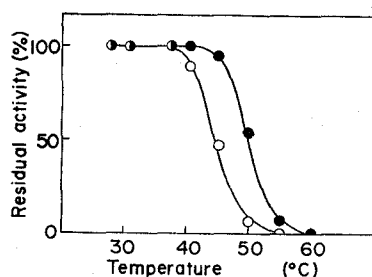


FIG. 2. Thermal Stabilities of Metaphosphate- and ATP-Dependent NAD Kinases in Cell Extracts of *B. ammoniagenes*.

Cell extracts (2.5 mg/ml of protein) equilibrated with 0.1 M Tris-HCl buffer (pH 7.0) containing 0.01 M $MgCl_2$ were heated at the indicated temperature for 5 min. After cooling, the reaction was started with the addition of other components, and the residual activities were determined. ○, metaphosphate-dependent NAD kinase; ●, ATP-dependent NAD kinase.

dent NAD kinase was more unstable than ATP-dependent NAD kinase.

3) Effect of freezing and thawing

Crude cell extracts were subjected to freezing and thawing, and the changes in metaphosphate- and ATP-dependent NAD kinase activities were examined. Metaphosphate-dependent NAD kinase lost 50 % of initial

activity by four cycles of freezing and thawing, though the ATP-dependent NAD kinase activity remained constant (Data not shown).

4) Metal ion effect

As shown in Table I, Mg^{2+} accelerated the metaphosphate-dependent NAD kinase reaction. So, the effect of Mg^{2+} , Mn^{2+} , and Ca^{2+} concentrations on metaphosphate-dependent NAD kinase activity was examined and compared with that of ATP-dependent NAD kinase activity (Figure 3).

Metaphosphate-dependent NAD kinase activity increased with increasing concentrations of divalent metal ions. On the other hand, the activity of ATP-dependent NAD kinase activity was inhibited by high ion concentrations.

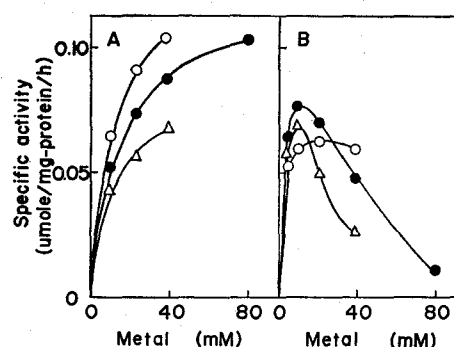


FIG. 3. Effect of Divalent Metal Ions on Metaphosphate (A)- and ATP (B)-Dependent NAD Kinase Activities in Cell Extracts of *B. ammoniagenes*. Reaction was carried out as described in MATERIALS AND METHODS, except that various metal ions were used at the indicated concentrations. ●, $MgCl_2$; ○, $CaCl_2$; △, $MnCl_2$.

5. Effect of phosphoryl donors on NAD phosphorylation by metaphosphate-dependent NAD kinase

As shown in Table VIII, of phosphoryl donors tested, only metaphosphate was a phosphoryl donor and trimetaphosphate or tetrametaphosphate were inert. Polyphosphates of different degree of condensation were not utilized as phosphoryl donors. p-

Nitrophenyl phosphate which is a good phosphoryl donor for phosphorylation of NAD by reverse reaction of acid phosphatase^{22,23} was also inert as a phosphoryl donor. The simultaneous incubation of two substrates, metaphosphate and ATP, remarkably increased the rate of NAD synthesis.

TABLE VIII. VARIOUS PHOSPHORYL DONORS FOR NAD PHOSPHORYLATION

Phosphoryl donor and concentration		Specific activity ^a
Pyrophosphate	5.0 mM	0
Tripolyphosphate	2.0 mg/ml	0
Polyphosphate	2.0 mg/ml	0
Trimetaphosphate	2.0 mg/ml	0
Tetrametaphosphate	2.0 mg/ml	0
Metaphosphate	0.5 mg/ml	0.054
	1.0 mg/ml	0.087
	2.0 mg/ml	0.071
ATP	5.0 mM	0.074
ATP (5.0 mM)+ Metaphosphate (1.0 mg/ml)		0.145
p-Nitrophenyl phosphate	5.0 mM	0

^a μ mol of NADP formed/mg of protein/h.

Reaction was carried out using cell extract of *B. ammoniagenes* under standard assay conditions, except for phosphoryl donors at the indicated concentrations.

DISCUSSION

In many microorganisms, NADP is synthesized by two enzymes. One of them is ATP-dependent NAD kinase which catalyzes the NAD phosphorylation using ATP as phosphoryl donor. The other is phototransferase, demonstrated by Ogata et al.^{22,23}). This enzyme phosphorylates NAD using p-nitrophenyl phosphate and produces NADP and its analogues.

In the course of the studies on the utilization of energy in phosphate polymers, I found an activity utilizing metaphosphate for NAD phosphorylation in *B. ammoniagenes* cell extracts. The distribution of NAD phosphorylating activity was investigated with

ATP and metaphosphate as phosphoryl donors. As shown in Table I, the ATP-dependent NAD kinase activity distributed in all microorganisms tested, whereas metaphosphate-utilizing activity was limited to a few genera. This pattern of distribution of metaphosphate-utilizing activities was also quite different from that of phosphotransferase activities reported by Ogata et al.²³). In fact, *B. ammoniagenes* did not utilize p-nitrophenyl phosphate for NAD phosphorylation (Table VIII).

The interest is whether the metaphosphate-utilizing enzyme is the same enzyme as ATP-dependent NAD kinase. I confirmed that these two NAD kinase activities were probably catalyzed by two different enzymes, and I called metaphosphate-utilizing enzyme as metaphosphate-dependent NAD kinase discriminating it from ATP-dependent NAD kinase from the following results. (1) The distribution patterns of the two kinase activities was different (Table III). (2) The two kinase activity ratios varied with cultural conditions, especially aeration (Table VI) and carbon sources (Table VII). This fact means that the metaphosphate-dependent NAD kinase is constitutive enzyme, and the ATP-dependent NAD kinase is an inducible one. (3) The stabilities of two kinases toward heat (Fig. 2) and freezing and thawing were different each other. (4) Optimal pH (Fig. 1) and metal ion requirement (Fig. 3) for reactions were different. (5) NADP synthesis increased remarkably by incubating the two substrates (metaphosphate and ATP) simultaneously (Table VIII). To verify the nonidentity of the two kinases in *B. ammoniagenes*, it is necessary to separate these two activities.

Of various properties of metaphosphate-dependent NAD kinase, the specificity for phosphoryl donor is the most interesting point. As shown in Table VIII, metaphosphate, which is a mixture of various ring phosphate polymers, was the only potent phosphoryl

donor of phosphate polymers tested. This fact indicates that ring phosphate polymers are more active than chain phosphate polymers in biological reactions. The determination of intrinsic phosphoryl donors in metaphosphate is indispensable for elucidation, not only of the role of metaphosphate-dependent NAD kinase in vivo, but also of the mechanism of utilization of these phosphoryl donors. Therefore, improved methods are now required for separation and isolation of phosphate polymers.

Thus, the results obtained indicate the possibility of the existence of a new NAD phosphorylating enzyme, other than ATP-dependent NAD kinase and phosphotransferase. Studies on phosphate polymer metabolism will be of importance not only for elucidation of the controlling mechanism of cellular energy supply, but also in the consideration of cellular regulation of NADP level.

SUMMARY

A new NAD kinase which synthesizes NADP from NAD and metaphosphate was found in some microorganisms. The activity of this enzyme, designated tentatively as metaphosphate-dependent NAD kinase, was detected in *Acetobacter*, *Achromobacter*, *Brevibacterium*, *Corynebacterium* and *Micrococcus* detected in *Escherichia*, *Proteus*, *Bacillus* and *Aerobacter* species. The metaphosphate-dependent NAD kinase activity of *B. ammoniagenes* IAM 1645 was not affected by culture conditions, though the ATP-dependent NAD kinase activity was did. The metaphosphate-dependent NAD kinase activity from *B. ammoniagenes* also differed from the ATP-dependent NAD kinase activity in optimal pH of reaction and stability in heating and in freezing and thawing. Of phosphate polymers tested, the potent phosphoryl donor was meta-

phosphate alone, and other chain and ring phosphate polymers of different degrees of condensation were not utilized by this enzyme.

1-2 A metaphosphate-dependent NAD kinase from
Brevibacterium ammoniagenes

INTRODUCTION

In the previous section, I described the existence of metaphosphate utilizing enzyme for NAD phosphorylation. The distribution pattern of this enzyme and enzymatic properties were different from those of ATP-dependent NAD kinase (EC 2.7.1.23) and phosphotransferase²³). To verify the nonidentity of metaphosphate-utilizing enzyme with above two enzyme activities, I isolated this enzyme from *B. ammoniagenes* cells having metaphosphate- and ATP-utilizing activities, but not phosphotransferase.

MATERIALS AND METHODS

purification of metaphosphate-dependent NAD kinase from B. ammoniagenes

In the following procedures, unless otherwise noted, centrifugation was carried out at 25,000 xg for 30 min, and 5.0 mM Tris-HCl buffer (pH 7.0) containing 0.5 mM NAD (Tris-NAD buffer) was used. All procedures were carried out at 0-4 °C.

1) *Preparation of cell extracts* *B. ammoniagenes* cells were grown on the nutrient Medium A described in previous section. The cells were harvested and washed once with 0.85 % NaCl solution. After being suspended in Tris-NAD buffer, cells were homogenated on a Dyno-Mill for 10 min, and the homogenate was centrifuged. The extracts thus obtained was fractionated with ammonium sulfate.

2) *1st ammonium sulfate fractionation* Ammonium sulfate (240 g) was added to the extracts (1000 ml, 18.3 g of protein). After stirring for 30 min, the mixture was centrifuged, and ammonium sulfate (63 g) was added to the supernatant. After stirring for 30 min, the precipitate was collected by centrifugation and dissolved in 80 ml of Tris-NAD buffer. This enzyme solution was dialyzed against the same buffer overnight.

3) *2nd ammonium sulfate fractionation* Ammonium sulfate (28 g) was added to the dialysate (100 ml, 8.75 g of protein) of 1st ammonium sulfate fraction and the precipitate separated by centrifugation was discarded. To the supernatant, ammonium sulfate (7.6 g) was added, and a second precipitate was collected by centrifugation and dissolved in 50 ml of Tris-NAD buffer and dialyzed against the same buffer overnight.

4) *DEAE-cellulose column chromatography* The dialyzed ammo-

nium sulfate fraction (75 ml, 1.43 g of protein) was applied to DEAE-cellulose column (5 x 60 cm) equilibrated with Tris-NAD buffer. The adsorbed protein was eluted with a linear 0-0.5 M KCl gradient in Tris-NAD buffer and the effluent was collected as 11 ml portions. The enzyme was eluted with 0.25 M KCl and the active fractions (tube No. 55-105) were pooled, concentrated ten fold by ultrafiltration (Amicon UM-10 membrane) and then dialyzed against Tris-NAD buffer overnight. The dialysate was concentrated again to 25 ml by ultrafiltration (Amicon UM-10 membrane).

5) *3rd ammonium sulfate fractionation* The enzyme solution (25 ml, 0.19 g protein) from the DEAE-cellulose column was fractionated into five fractions with ammonium sulfate (Fig. 1). The 1st fraction was obtained as follows. The enzyme solution was brought to 20 % saturation by adding 2.8 g of ammonium sulfate slowly with stirring. After 30 min, the precipitate was collected by centrifugation. 2nd fraction was obtained by adding 1.5 g (30 % saturation) of ammonium sulfate to the supernatant of 1st fraction, and by collecting the precipitates formed after 30 min. In the same manner as employed in the case of 2nd fraction, 3rd, 4th and 5th fractions were obtained successively adding ammonium sulfate to the respective supernatants. The amount of ammonium sulfate added for obtaining 3rd, 4th and 5th fractions was 1.5 g (40 % saturation), 1.6 g (50 % saturation) and 3.4 g (70 % saturation), respectively. The precipitates thus obtained were dissolved in 2.0 ml of Tris-NAD buffer and dialyzed against the same buffer overnight. The enzymatic activities of each fraction was determined, and 4th fraction was saved for the further purification.

6) *Sephadex G-150 column chromatography* Ammonium sulfate fraction (3.2 ml, 21 mg of protein) was applied to a Sephadex G-150 column (2.5 x 90 cm) equilibrated with Tris-NAD buffer. Protein was eluted with buffer and the effluent was collected as 4.0 ml portions. The active fractions (tube No. 55-67) were pooled and concentrated five fold by ultrafiltration (Amicon UM-10 membrane).

7) *Hydroxylapatite column chromatography* The concentrated enzyme solution (10 ml, 3.7 mg of protein) from the Sephadex G-150 column was applied to a hydroxylapatite column (1.2 x 15 cm) equilibrated with 1.0 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM NAD. Enzyme was eluted with a linear 1.0-200 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM NAD, and the effluent was collected as 2.0 ml portions. The enzyme was eluted at about 0.07 M potassium phosphate buffer (pH 7.0). The active fractions (tube No. 17-22) were pooled and dialyzed against 5.0 mM Tris-HCl buffer (pH 7.0) overnight. The dialysate was concentrated to 1.4 ml by ultrafiltration (Amicon UM-10 membrane) and stored at -20 °C.

Polyacrylamide gels Disc gel electrophoresis of native enzyme was performed on 6 cm gels of 7.0 % acrylamide, 0.2 % bisacrylamide, polymerized with N,N,N',N'-tetramethylethylenediamide and 0.1 % ammonium persulfate. Electrophoresis was performed for 90 min at 100 mV (2 mA/tube) in 25 mM Tris, 100 mM glycine buffer (pH 8.0). Gels were stained for protein with Coomassie blue²⁷.

Gel filtration Molecular weight determination was performed on a column of Sephadex G-150 (1.0 x 90 cm) by the methods of Andrews²⁸. The protein was eluted with 5.0 mM Tris-HCl buffer (

pH 7.0) at a flow rate of 0.5 ml/min at 4 °C.

Assay of metaphosphate-dependent NAD kinase activity The metaphosphate-dependent NAD kinase activity was determined in the following reaction mixture; 10 mg metaphosphate, 2 μ mol NAD, 10 μ mol $MgCl_2$, 50 μ mol Tris-HCl buffer (pH 7.0), and 10 μ g of enzyme in a volume of 1.0 ml. The reaction was allowed to proceed for 20 min at 37 °C, and NADP formed in reaction mixture was determined by enzymatic method²⁵. In some experiments, 5 μ mol ATP was used as phosphoryl donor instead of metaphosphate. In experiments examining inhibition by reduced NADP (NADPH), the reaction was terminated by addition of 0.1 ml of 0.5 M HCl. After 5 min at room temperature, 0.1 ml of 0.75 M Tris base was added and the residual NADP was assayed. Specific activity was expressed in terms of nmoles of NADP formed per mg of protein per hour. Protein was determined by absorbance measurement at 280 nm ($A_{1\%} = 10.5 \text{ cm}^{-1}$).

Chemicals AMP, ADP and ATP were obtained from Kojin Co., Tokyo, Japan. NAD, NADP, NADH and NADPH were obtained from Oriental Yeast Co., Tokyo, Japan. ADPG, ADPR, AMN and FAD were purchased from Sigma Chemical Co., St. Louis MO. Trimetaphosphate, tetrametaphosphate and tripolyphosphate were also purchased from Sigma Chemical Co., St. Louis MO. Metaphosphate and polyphosphate were obtained from Katayama Chemical Industries Co., Osaka, Japan. The other chemicals were all analytical grade reagents.

RESULTS

Purification of metaphosphate-dependent NAD kinase from B. ammoniagenes

The metaphosphate-dependent NAD kinase and ATP-dependent NAD kinase activities were successfully separated by 3rd ammonium sulfate fractionation (Fig. 1). In this experiment, NAD phosphorylating activities appeared in two fractions. One precipitated by 30-40 % saturation with ammonium sulfate and efficiently used ATP as phosphoryl donor. The other active fra-

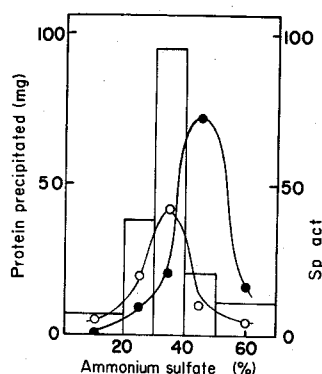


FIG. 1. Separation of Metaphosphate-dependent NAD Kinase and ATP-dependent NAD Kinase.

The partially purified metaphosphate-dependent NAD kinase fraction from DEAE-cellulose column chromatography was further fractionated by ammonium sulfate with the saturation indicated in figure. This figure shows the distribution of activities of two NAD kinases in the presence of metaphosphate (●) and ATP (○) as phosphoryl donors.

TABLE I. SUMMARY OF PURIFICATION PROCEDURES

Step	Preparation	Total protein (mg)	Sp act	Yield (%)	Ratio of Sp act ^a
1	Original extracts	18300	1.08	100	0.54
2	1st ammonium sulfate	8750	2.48	109	1.22
3	2nd ammonium sulfate	1430	7.78	56.1	1.88
4	DEAE-cellulose	190	46.7	44.9	2.65
5	3rd ammonium sulfate	21.0	139	14.6	2.85
6	Sephadex G-150	3.96	418	8.19	3.01
7	Hydroxylapatite	2.49	519	6.18	2.82

^a Ratio: specific activity for metaphosphate/specific activity for ATP. The details of purification were described in METHODS AND MATERIALS.

ction was precipitated by 40-50 % saturation and efficiently used metaphosphate as phosphoryl donor. As summarized in Table I, metaphosphate-dependent NAD kinase was purified about 500 fold from the extracts with recovery of 6.2% of the initial activity. The purified metaphosphate-dependent NAD kinase preparation showed a single band on polyacrylamide gel electrophoresis (Fig. 2). The molecular weight of this enzyme was determined to

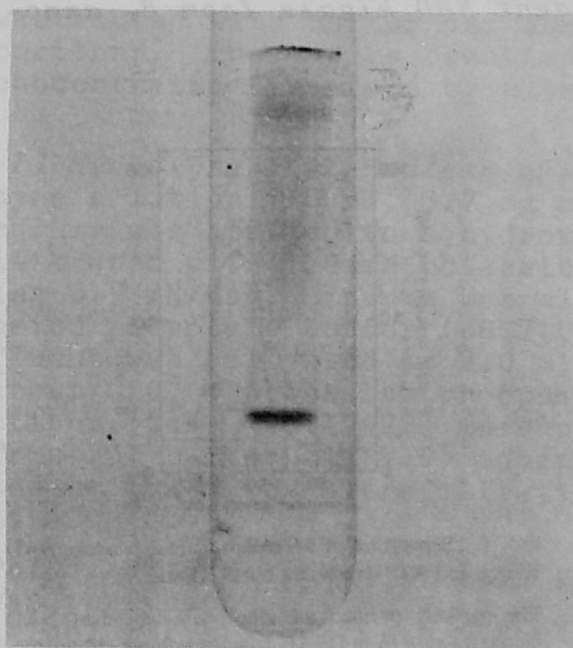


FIG. 2. Disc Gel Electrophoresis of Metaphosphate-dependent NAD Kinase.

The methods for the electrophoresis were described in MATERIALS AND METHODS. The direction of migration is from the cathode (top of photo) to the anode.

be 9.0×10^4 by gel filtration on Sephadex G-150 (Fig. 3).

The subunit structure of this enzyme was not determined, since the polyacrylamide gel electrophoresis in the presence of sodium dodesyl sulfate failed to separate the enzyme more than one components.

Enzymatic properties of metaphosphate-dependent NAD kinase

1) *Phosphoryl donor and acceptor specificity*

Phosphoryl donor specificity of metaphosphate-dependent

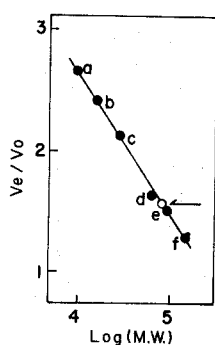


FIG. 3. Determination of Molecular Weight of Metaphosphate-dependent NAD Kinase.

Molecular weight of metaphosphate-dependent NAD kinase was determined by gel filtration on Sephadex G-150. Marker proteins: a, cytochrome c; b, myoglobin; c, chymotrypsinogen A; d, albumin; e, hexokinase; f, γ -globulin. The position of metaphosphate-dependent NAD kinase was shown by arrow. V_o and V_e are void volume of column and elution volume of protein, respectively.

NAD kinase was examined with purified enzyme (Table II). Among the phosphate polymers tested, metaphosphate was specifically used by this enzyme, and other ring or chain phosphate polymers were not. However, intrinsic phosphoryl donors in metaphosphate could not be determined, since the metaphosphate was a mixture of various ring phosphate polymers. In addition to metaphosphate, this enzyme also

utilized ATP as phosphoryl donor. *p*-Nitrophenylphosphate, a substrate utilized by acid phosphatase for the phosphorylation of NAD, was not utilized by metaphosphate-dependent NAD kinase.

Phosphoryl acceptor specificity of this enzyme was also investigated using metaphosphate as phosphoryl donor (Table III).

Among the compounds tested, only NAD was phosphorylated by this enzyme, and the phosphorylation product was NADP only. This was confirmed by the following results: (1) R_f values of the product on several paper chromatograms with different solvent systems were the same as that of authentic NADP, (2) the product was completely reduced by the reaction of glucose

TABLE II. PHOSPHORYL DONOR SPECIFICITY OF METAPHOSPHATE-DEPENDENT NAD KINASE
Activities were assayed under standard assay conditions except that the various phosphoryl donors were used at the indicated concentrations.

Phosphoryl donor	Concentration	NADP formed (mm)
Pyrophosphate	5.0 mm	0
Triphosphate	1.0 mg/ml	0
	10 mg/ml	0
Polyphosphate	1.0 mg/ml	0
	10 mg/ml	0
Trimetaphosphate	1.0 mg/ml	0
	10 mg/ml	0
Tetrametaphosphate	1.0 mg/ml	0
	10 mg/ml	0
Metaphosphate	1.0 mg/ml	0.16
	10 mg/ml	0.11
ATP	0.5 mm	0.03
	2.0 mm	0.07
<i>p</i> -Nitrophenylphosphate	5.0 mm	0
	10 mm	0

TABLE III. PHOSPHORYL ACCEPTOR SPECIFICITY OF METAPHOSPHATE-DEPENDENT NAD KINASE

Phosphoryl acceptor (5 mM)	R _f values of phosphoryl acceptor				R _f values of phosphorylated compounds			
	I	II	III	IV ^a	I	II	III	IV
Adenosine	0.94	0.71	0.14	—	N.D. ^b	N.D.	N.D.	—
AMP	0.77	0.54	0.34	—	N.D.	N.D.	N.D.	—
ADP	0.67	0.42	0.39	—	N.D.	N.D.	N.D.	—
ADPG	0.62	0.61	0.41	—	N.D.	N.D.	N.D.	—
ADPR	0.65	0.64	0.38	—	N.D.	N.D.	N.D.	—
AMN	0.94	0.79	0.10	—	N.D.	N.D.	N.D.	—
NAD	0.75	0.48	0.30	—	0.63 (0.62)	0.30 (0.31)	0.46 (0.47) ^c	—
FAD	—	—	—	0.14	—	—	—	N.D.

^a Following solvent systems were used for the detection of phosphorylated compounds: I, isobutyrate-0.5 N NH₄OH (5:3 v/v); II, 95% ethanol-1.0 M CH₃COONa (7:3 v/v); III, saturated (NH₄)₂SO₄-0.1 M CH₃COONa-isopropanol (40:10:1 v/v); IV, buthanol-CH₃COOH-H₂O (4:1:1 v/v).

^b Phosphorylated compounds were not detected.

^c R_f values of authentic NADP.

Purified enzyme solution was dialyzed against 5 mM Tris-HCl buffer (pH 7.0) overnight, and then used for the reaction.

-6-phosphate dehydrogenase or isocitrate dehydrogenase.

2) pH optimum and pH stability

The pH optimum of metaphosphate-dependent NAD kinase reaction was around 6.0, and this enzyme was stable at the pH range of 5.0 to 6.0 (Fig. 4). The same pattern of pH-activity and pH-stability profiles were obtained in both cases of phosphoryl donors, metaphosphate and ATP.

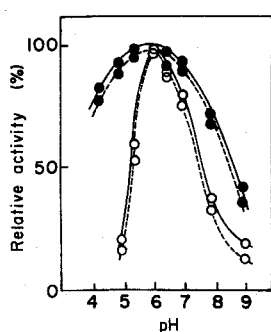


FIG. 4. pH Optimum and pH Stability of Metaphosphate-dependent NAD Kinase.

In the studies of pH optimum, reaction was carried out under various pH values in the presence of metaphosphate (—○—) and ATP (—○—). In the studies of pH stability, enzyme was incubated at 40°C for 10 min in 0.02 M buffer with various pH values. Aliquots of incubation mixture were adjusted to pH 7.0, and assayed the residual activity using metaphosphate (—●—) or ATP (—●—). pH was adjusted to the indicated value with Tris-maleate buffer.

3) Effect of temperature on stability and activity

The effects of temperature on stability and activity of metaphosphate-dependent NAD kinase were examined using metaphosphate and ATP as phosphoryl donors (Fig. 5).

This enzyme was rapidly inactivated above 40 °C and completely lost the activity at 57 °C for 10 min. But this enzyme was slightly protected from the thermal inactivation by

the addition of NAD (Fig. 5A).

The optimal temperature for this enzyme reaction was around 50 °C (Fig. 5B).

4) Metal requirement

Divalent metal ions were indispensably required for this enzyme reaction as shown in previous section. Of the metal ions tested, Ca^{2+} , Mg^{2+} and Mn^{2+} were more potent activator than others. The activity was strongly dependent on the concentration of these metal ions and increased with the concentration (Table IV). But the reaction did not proceed at the concentration above 70 mM Mg^{2+} , since the precipitate was formed.

5) Effect of inhibitors on enzyme activity

The effect of various inhibitors on metaphosphate-dependent NAD kinase activity was investigated. (Table V). This enzyme

TABLE IV. EFFECT OF METAL IONS ON METAPHOSPHATE-DEPENDENT NAD KINASE ACTIVITY

Metals	Concentration (mM)	Relative activity (%) ^a
None		0
MgCl_2	5	100
	30	140
	60	176
MnCl_2	5	100
	30	135
CaCl_2	5	120
	30	165
CoCl_2	5	1.7
NiCl_2	5	0.9
ZnCl_2	5	4.6
HgCl_2	0.5	0

^a Relative activities were based on the 100% activity in the presence of 5 mM MgCl_2 .

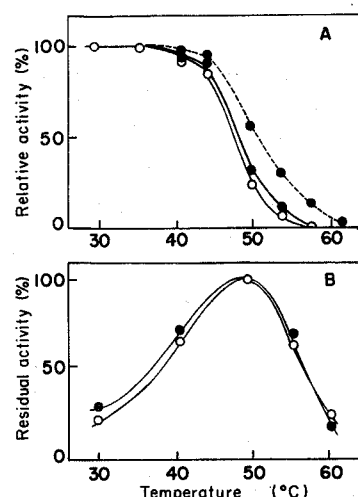


FIG. 5. Thermal Stability of Metaphosphate-dependent NAD Kinase (A) and Optimal Temperature for Activity (B).

A; The enzyme solution equilibrated with 5 mM Tris-HCl buffer (pH 7.0) was treated at the indicated temperature for 10 min with (---) or without (—) 5.0 mM NAD, and the residual activities were assayed using metaphosphate (●) or ATP (○).

B; In the studies of temperature optimum, reaction was carried out at the indicated temperature for 2 min using metaphosphate (●) or ATP (○) as phosphoryl donors.

reaction was inhibited by AMP, ADP, NADH, NADPH and sulfhydryl reagents such as HgCl_2 and p-chloromercuribenzoate. Of these inhibitors tested, inhibition by AMP, ADP and NADPH were competitive with NAD. On the other hand, adenosine, and ring or chain phosphate polymers of low molecular weight showed no inhibition.

TABLE V. EFFECT OF VARIOUS INHIBITORS ON METAPHOSPHATE-DEPENDENT
NAD KINASE ACTIVITY
Reaction was carried out under standard assay conditions with or without various inhibitors.

Addition (mM)		Relative activity (%)	Type of inhibition	K_i (mM)
None		100		
Adenosine	1.0	100		
	10	100		
AMP	1.0	95.0	Competitive	9.80
	6.0	71.0		
ADP	0.1	75.4	Competitive	0.58
	0.2	67.1		
ADPG	0.5	65.4	Competitive	0.43
ADPR	0.5	66.2		
NADH	0.5	72.1		
NADPH	0.5	53.3		
Iodoacetate	1.0	88.0		
HgCl ₂	0.5	0		
<i>p</i> -Chloromercuribenzoate	0.1	0		
Pyrophosphate	5.0	97.8		
Triphosphate	2.0	95.8		
Trimetaphosphate	2.0	97.5		
Tetrametaphosphate	2.0	98.6		

6) Kinetics

The effect of metaphosphate concentration on reaction rate was examined. The reaction proceeded efficiently at concentration up to 1.0 mg/ml. Above this concentration, metaphosphate inhibited the reaction (Fig. 6). The K_m values for NAD with 1.0 mg/ml and 10 mg/ml of metaphosphate were 3.0×10^{-4} M and 6.2×10^{-4} M, respectively. On the other hand, the K_m value for NAD in the presence of ATP as phosphoryl donor was 2.3×10^{-3} M. The K_m values for intrinsic substrate in metaphosphate, however, could not be determined, since I could not isolate these substrates in pure state.

The K_m value for ATP was 6.7×10^{-3} M.

The reaction proceeded com-

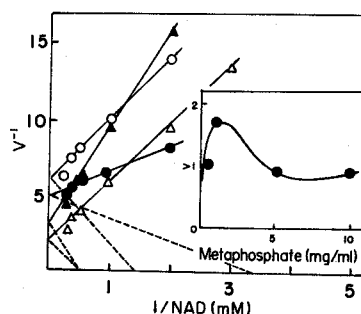


FIG. 6. Dependence of Reaction Rate on NAD and Metaphosphate (Inset Figure) Concentrations.

Dependence of reaction rate on NAD concentration was determined in the presence of metaphosphate or ATP. Velocities are in arbitrary units. Symbols: metaphosphate 1.0 mg/ml (●), 10 mg/ml (○); ATP 1.0 mM (▲), 2.0 mM (△).

pletely in the direction of NADP formation, and about 25 mg of metaphosphate was required for the conversion of 1.0 μmol of NAD to NADP (Fig. 7).

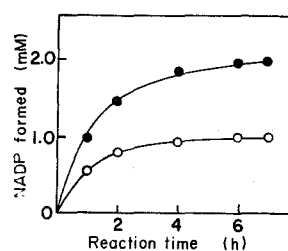


FIG. 7. Time Course of Metaphosphate-dependent NAD Kinase Reaction.

Reaction was proceeded under standard assay conditions, except for the concentration of metaphosphate: 25 mg/ml (○); 50 mg/ml (●).

DISCUSSION

In many microorganisms, NADP has been synthesized by two enzymes, ATP-dependent NAD kinase and phosphotransferase (*i.e.* reverse reaction of acid phosphatase). However, in the course of the studies on the utilization of energy in phosphate polymers, I recognized an activity utilizing metaphosphate for NAD phosphorylation in *B. ammoniagenes*.

It seems certain that the metaphosphate-utilizing activity for NAD phosphorylation is different from the phosphotransferase activity, since the distribution pattern of metaphosphate-utilizing activity was quite different from that of phosphotransferase activity reported by Ogata et al.²³⁾. Furthermore, metaphosphate-utilizing enzyme for NAD phosphorylation differed from phosphotransferase in some enzymatic properties. For example, the reaction product of metaphosphate-utilizing enzyme was NADP only, though the phosphotransferase reaction produces biologically inactive NADP analogues in addition to NADP²²⁾.

Therefore, the most interesting point was whether or not the metaphosphate-utilizing enzyme for NAD phosphorylation is the same enzyme as an ATP-dependent NAD kinase. So, I attempted to purify the former enzyme from *B. ammoniagenes*.

In analogy with an ATP-dependent NAD kinase of various sources, the purified metaphosphate-utilizing enzyme required metal ions for activity, and was inhibited by adenosine nucleotides or reduced pyridine nucleotides. This enzyme was also inhibited by sulfhydryl reagents. Furthermore, contrary to my expectation, this enzyme used ATP besides metaphosphate. These results make it difficult to discriminate this enzyme from an ATP-dependent NAD kinase. The possibility that the isolated metaphosphate-utilizing enzyme is a subunit of original ATP-dependent NAD kinase has not been excluded.

From the following facts, however, it was strongly suggested that this enzyme is different from an ATP-dependent NAD kinase. (1) the distribution pattern of two enzyme activities is different (section 1-1) (2) these two enzymes in crude extracts showed quite different activity with respect to pH and temperature (section 1-1) (3) the activity ratio of two kinases changed by culture conditions (section 1-1) (4) these two enzyme activities were separated by ammonium sulfate fractionation (Fig. 1) (5) in the course of purification after ammonium sulfate fractionation, the ratio of metaphosphate *vs.* ATP dependent NAD kinase activity remained constant (6) the molecular weight of metaphosphate-utilizing enzyme was smaller in comparison with that of ATP-dependent NAD kinase of sea urchin egg (3.1×10^5)²⁹⁾, pigeon liver (2.7×10^5)³⁰⁾, *Azotobacter vinelandii* (1.3×10^5)³¹⁾ and rabbit liver (1.36×10^5)³²⁾. Therefore, I considered this enzyme to be different from ATP-dependent NAD kinase, and I designated this enzyme as metaphosphate-dependent NAD kinase.

This enzyme possibly is involved in the regulation of intracellular NADP level and in the metabolism of phosphate polymers

in microorganisms. Furthermore, it is also clear that the certain phosphate polymers could perform the functions usually inherent to ATP, since many enzymes utilizing phosphate polymers for phosphorylation of biological molecules are known in microorganisms. Such enzymes are polyphosphate kinase¹³⁾, polyphosphate glucokinase¹⁷⁾, polyphosphate fructokinase¹⁸⁾, polyphosphate-AMP phosphotransferase¹⁹⁾ and metaphosphate-dependent NAD kinase presented in this section. In addition to these enzymes, I found the metaphosphate-utilizing activity for phosphorylation of CMP to CTP in extracts of *Enterobacter aerogenes* (data not shown here). Other enzymes utilizing certain phosphate polymers as phosphoryl donor are expected to exist in microorganisms. Therefore, it is necessary to study not only the biosynthetic and degradative pathways of phosphate polymers, but also the content of them in microorganisms for the elucidation of the basic functions of phosphate polymers with respect to the energy supply.

SUMMARY

The enzyme utilizing metaphosphate for NAD phosphorylation was purified 500-fold from *B. ammoniagenes* and its properties were studied. The isolated enzyme appeared homogeneous on disc gel electrophoresis: its molecular weight was determined to be 9.0×10^4 by gel filtration. This enzyme specifically phosphorylated NAD at the optimum pH at 6.0. Of phosphoryl donors tested, metaphosphate was most effective for the reaction, and ATP was less effective. The activity was inhibited by AMP, ADP or reduced pyridine nucleotides. The enzyme did not exhibit catalytic activity in the absence of divalent cation. I concluded that the enzyme phosphorylating NAD in the presence of metaphos-

phate is distinct from ATP-dependent NAD kinase, and tentatively designated it metaphosphate-dependent NAD kinase.

1-3 Continuous production of NADP by immobilized *Brevibacterium ammoniagenes* cells

INTRODUCTION

NADP has been produced by extraction from microbial cells. From an industrial standpoint, however, this method is not always satisfactory since it requires tedious and intricate procedures in addition to the low content of NADP in microbial cells.

To overcome these disadvantages, Uchida et al.³³⁾ studied a method for continuous production of NADP from NAD and ATP using immobilized *Achromobacter acris* cells. In this case, however, the ATP-degrading activities should be completely inactivated to obtain NADP in a high yield, and also the utilization of ATP was not economical.

Thus in order to develop a more efficient method for NADP production, I have studied the utilization of metaphosphate-dependent NAD kinase in *Brevibacterium ammoniagenes* cells.

This section deals with the properties of immobilized *B. ammoniagenes* cells and its application for the continuous production of NADP.

MATERIALS AND METHODS

Microorganisms and cultivation *Brevibacterium ammoniagenes* IAM 1645 was used as the source of metaphosphate-dependent NAD kinase. The cultivation of cells was the same as described in the section 1-1.

Preparation of immobilized cells The procedure for immobilizing cells in a polyacrylamide gel lattice was the same as described by Chibata et al.³⁴. By this method, 0.3 g(wet weight) intact cells was immobilized in 1.0 ml gel.

Standard assay conditions of metaphosphate-dependent NAD kinase activity Unless otherwise noted the following reaction system was employed. The immobilized cells (3.0 ml) or intact cells

(0.9 g) were incubated in 10 ml of mixture containing 50 mg/ml metaphosphate, 2.1 mM NAD, 40 mM MgCl_2 , 100 mM Tris-HCl buffer (pH 7.0) at 37 °C with shaking. The amount of NADP formed in the reaction mixture was determined by an enzymatic method^{2,5}. A sonicate of intact cells and an immobilized cell homogenate were prepared by the method of Nabe et al.^{3,5} and they were also used as enzyme sources. Metaphosphate-dependent NAD kinase activity was expressed as mM NADP formed per 3 h or μmol NADP formed/g-cells/h.

RESULTS

Activation of immobilized cells

In general, it is necessary to remove the cell membrane barrier so that the phosphate esters are able to penetrate into the cells and to make contact with the enzyme. Therefore, the treatment conditions for increasing the permeation of the cell membrane were investigated. As a result, treatment with organic solvents or detergents was found to be effective to increase apparent metaphosphate-dependent NAD kinase activity of immobilized cells. (Table I). Among the treatments, acetone treatment of immobilized cells was considered to be the most effective and easily applicable. Then the conditions for acetone treatment were studied and determined as follows. 10 ml of immobilized cells were incubated in 100 ml acetone at room temperature for 30 min under stirring. Intact cells (3 g) were also treated in the same manner. A summary of metaphosphate-dependent NAD kinase activity of *B. ammoniagenes* cells after acetone treatment is shown in Figure 1. The intrinsic activity of

TABLE I
Activation of Immobilized Cells under Various Conditions

Treatment conditions	Activity ^a after treatment for	
	30 min	60 min
100% Acetone	0.185	0.231
10% Toluol	0.151	0.183
10% Butanol	0.060	0.115
0.04% Cation-S ^b	0.101	0.122
0.08% Cation-S	0.116	0.182
0.05% SDS ^c	0.065	0.105
0.10% SDS	0.091	0.153
0.85% NaCl	0.004	0.011

^a Immobilized cells (3.0 g) were incubated in 100 ml solutions indicated in the table for 30 and 60 min at room temperature under stirring. Activities were assayed under standard conditions as described in Materials and Methods. Activities were expressed as mM NADP formed/3 hr.

^b Lauryl trimethyl ammonium chloride.

^c Sodium dodecyl sulfate.

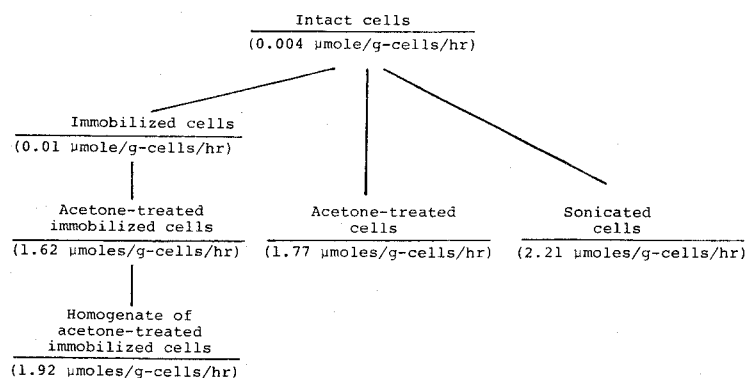


Fig. 1. Comparison of metaphosphate-dependent NAD kinase activities of various cell preparations.

metaphosphate-dependent NAD kinase in cells was estimated from the activity of thoroughly sonicated cells. The activity of this sonicate was 2.21 $\mu\text{mol/g-cells/h}$. On the other hand, the activity of immobilized cells after acetone treatment was 1.62 $\mu\text{mol/g-cells/h}$. After being homogenated, acetone-treated immobilized cells showed higher activity, 1.92 $\mu\text{mol/g-cells/h}$. These values mean about 90 % activity yield calculated from the activity of sonicated cells. This result indicates that the polyacrylamide gel is a barrier for transport of substrates and/or products.

Unless otherwise noted, in the following experiments, the terms

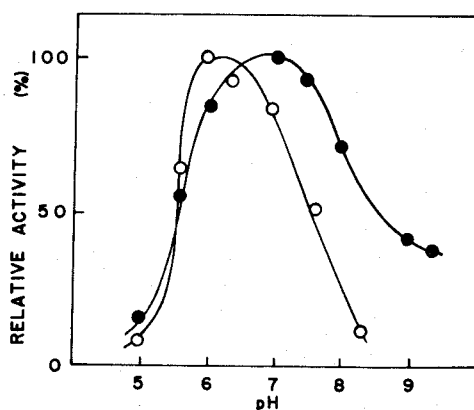


Fig. 2. Effect of pH on metaphosphate-dependent NAD kinase activity of intact cells and immobilized cells. Reaction conditions were the same as described in Materials and Methods except that Tris-maleate buffer was used. (o) Intact cells; (●) Immobilized cells

intact cells and immobilized cells represent both cell preparations activated with acetone.

Enzymatic properties of metaphosphate-dependent NAD kinase in immobilized cells

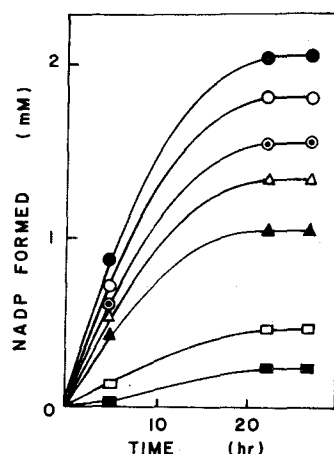


Fig. 3. Effect of metaphosphate concentration on NADP production by immobilized cells. Reaction conditions were the same as described in Materials and Methods except for metaphosphate concentration. Metaphosphate concentrations were: (■) 1.6 mg/ml; (□) 3.2 mg/ml; (▲) 6.4 mg/ml; (○) 10 mg/ml; (◐) 20 mg/ml; (●) 50 mg/ml; (△) 100 mg/ml.

In order to establish the most suitable conditions for continuous production of NADP, the enzymatic properties of immobilized cells were examined, and compared with those of intact cells.

Figure 2 shows the pH-rate profile for phosphorylation of NAD by immobilized cells and intact cells. The immobilized cells showed a maximum activity at pH 7.0, while the maximum of intact cells was at pH 6.0.

The effect of temperature on stabilities of metaphosphate-dependent NAD kinase was examined by incubating the immobilized cells (3.0 ml) or intact cells (0.9 g) in 10 ml of 5.0 mM Tris-HCl buffer (pH 7.0) at 45°C for several minutes. As a result,

TABLE II
Reuse of Immobilized *B. ammoniagenes* Cells

Times	Activity ^a	
	intact cells	immobilized cells
1	100	100
2	62	98
3	48	92
4	32	93
5	28	89

a) Relative activity(%). Metaphosphate-dependent NAD kinase activity was determined as described in Materials and Methods. Intact cells and immobilized cells were washed with 0.85 % NaCl before the addition of new substrate solution.

the metaphosphate-dependent NAD kinase in immobilized cells was shown to be more stable than that of intact cells. The immobilized cells retained 50 % of their initial activity, even after 40 min treatment. On the other hand, intact cells completely lost their activity by treatment for 40 min. The decrease in activity followed first-order kinetics, and

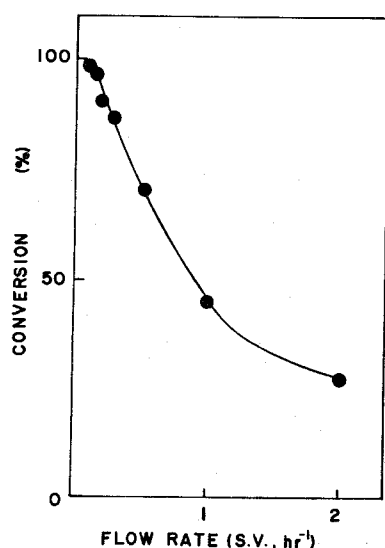


Fig. 4. Effect of flow rates on NADP production by immobilized cell column. Column (1 × 11 cm) packed with 8.0 g immobilized cells received the substrate solution (2.1mM NAD, 50 mg/ml metaphosphate, 40mM MgCl₂, pH 6.7) from the bottom of the column at various flow rates at 36.5°C. Activity of column was checked by determining the NADP formed in effluent.

the rate of activity loss was reduced by immobilization to $\frac{1}{2}$ that of intact cells. Figure 3 shows the effect of metaphosphate concentration on NADP formation by immobilized cells. Fifty mg metaphosphate were required for the conversion of 2.1 μ mol NAD to NADP. The metaphosphate-dependent NAD kinase of immobilized cells required metal ions for activity as shown in section 1-1. Therefore, the effect of various metal ions on NADP production was investigated. Of the metal ions tested, Mg²⁺ was the most effective activator of metaphosphate-dependent NAD kinase reaction, and the activities increased with the concentration of Mg²⁺ up to 60 mM. Other than Mg²⁺, Mn²⁺, Cu²⁺, Co²⁺, Ni²⁺ and Fe²⁺ were also effective for the activation of this enzyme reaction, although the data are not shown here.

Table II shows the metaphosphate-dependent NAD kinase activity of immobilized cells and intact cells after repeated use. It was found that the enzyme activity of immobilized cells was stable and was not decreased by repeated use.

Continuous production of NADP by immobilized cell column

Since the superiority of immobilized cells over intact cells was confirmed, the conditions for the continuous production of

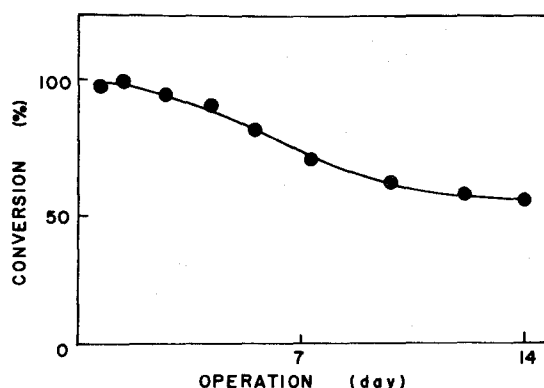


Fig. 5. Continuous production of NADP by the immobilized cell column. Substrate solution indicated in Figure 4 was passed through the column at a flow rate of $SV = 0.1 \text{ hr}^{-1}$ at 36.5°C . Other column conditions were the same as described in Figure 4.

NADP was investigated using an immobilized cell column. Figure 4 shows the relationship between flow rates of substrate solution and NADP produced in effluent. Phosphorylation of NAD to NADP was nearly complete at a flow rate below space velocity (SV) = 0.1 h^{-1} . In this experiment, Tris-HCl buffer was omitted from the substrate solution, since metaphosphate had a strong buffer action.

The operational stability of the immobilized cell column was investigated by passing the substrate solution continuously at $SV = 0.1 \text{ h}^{-1}$. As shown in Figure 5, NADP was continuously produced in a high yield by this column. The half-life of this column was estimated to be about eight days. On the other hand, the half-life of intact cells calculated from the data of Table II was about 10 h.

DISCUSSION

In previous sections 1-1 and 1-2, I revealed that metaphosphate-dependent NAD kinase is very promising for production of NADP, since the metaphosphate, phosphoryl donor of this enzyme, is readily available in much lower price than ATP, and NAD is completely converted to NADP. These advantages suggested the possibility of developing an efficient method to produce NADP. Accordingly, I investigated the continuous production of NADP using

the immobilized cell column having the metaphosphate-dependent NAD kinase activity and succeeded to produce NADP in good yield.

Of the conditions investigated, activation of immobilized cells and concentration of Mg^{2+} and metaphosphate were important in producing NADP in a high yield. The activation by acetone treatment seems to be due to the removal of membrane barriers for substrate and/or product transport (Fig. 1). The concentration of Mg^{2+} and metaphosphate have a great influence on the production rate of NADP.

The effect of Mg^{2+} may be explained as follows. Mg^{2+} accelerates the degradation of phosphate polymers (metaphosphate), as shown by Thilo^{36,37}), and the phosphoryl donors for metaphosphate-dependent NAD kinase are sufficiently supplied. This makes it difficult to determine not only the stoichiometry of phosphate transfer in the metaphosphate-dependent NAD kinase reaction, but also the content and degree of condensation of intrinsic phosphoryl donors in metaphosphate.

Under the conditions employed for NADP production, the amount of NADP produced was proportional to the metaphosphate concentration up to 10 mg/ml, and about 7 mg of metaphosphate was required for the production of 1 μ mol NADP. The study on intrinsic phosphoryl donors of metaphosphate-dependent NAD kinase in metaphosphate is indispensable not only for the efficient production of NADP, but also for the elucidation of reaction mechanism of this phosphorylation.

SUMMARY

Whole cells of *Brevibacterium ammoniagenes* IAM 1645 having the metaphosphate-dependent NAD kinase activity were successfully

immobilized in polyacrylamide gel lattice. The immobilized cells were activated by treatment with organic solvents or detergents. The pH optimum of immobilized cells for the production of NADP was 7.0, and divalent metal ions were required to maintain the elevated activity of metaphosphate-dependent NAD kinase. Highly pure NADP was continuously produced in high yield by the immobilized cell column. The half-life of this column was about eight days.

Section 2 G-6-P production with phosphate polymers

2-1 Properties of polyphosphate glucokinase in *Achromobacter butyrii*

INTRODUCTION

In most microorganisms, glucose is phosphorylated to G-6-P by ATP-dependent glucokinase or hexokinase. Only this route has been thought to be involved in glucose assimilation.

However, in recent years, many experiments have shown that some hydrolytic enzymes catalyze the transfer of phosphoryl group of certain compounds to glucose. For example, Morton³⁸⁾ showed alkaline phosphatase catalyzed the phosphoryl group transfer between creatine phosphate and glucose to give G-6-P. Another type of reaction catalyzed by non-specific acid phosphatase was demonstrated by Saif et al.³⁹⁾. In this case, G-6-P was formed from p-nitrophenyl phosphate and glucose. The transfer reactions catalyzed by these phosphatases were shown to be reverse reaction under the unusual conditions, such as extremely high or low pH.

In addition to the above enzymes, G-6-P was also formed by polyphosphate glucokinase. This enzyme was first demonstrated by Szymona et al.^{16,17,18,40)} in acetone-treated cells of *Mycobacterium phlei*. They also showed that this enzyme was different from the ATP-dependent glucokinase. The characteristic of this enzyme is that it utilizes certain phosphate polymers as phosphoryl donors in place of ATP. This fact indicates that G-6-P could be produced economically because such phosphate polymers are readily available at lower prices than ATP. Thus, I investigated to apply this enzyme for production of G-6-P.

In this section, I describe the distribution of polyphosphate glucokinase in microorganisms, and the properties of this enzyme in *Achromobacter butyri*.

MATERIALS AND METHODS

Cultivation of microorganisms All microorganisms except for acetic acid bacteria were cultivated in the Medium A described in section 1-1 in Chapter I. Acetic acid bacteria were grown in the medium described by Foda and Vaughn⁴¹. Cultivation was carried out under reciprocal shaking at 30 °C for 20 h in 5dl Sakaguchi flasks with 100 ml of medium.

Preparation of cell extracts Preparation of cell extracts were carried out as described in section 1-1 in Chapter I. Protein was determined by the method of Lowry et al.²⁶.

Determination of enzyme activity Unless otherwise noted, the following methods were used for the determination of polyphosphate glucokinase activity. The reaction was conducted at 37 °C for 10 min in a mixture of 30 mM glucose, 20 mg/ml metaphosphate, 5.0 mM MgCl₂, 100 mM Tris-HCl buffer (pH 7.0) and cell extracts (0.5-1.0 mg/ml of protein). For the assay of ATP-dependent glucokinase activity, 5.0 mM of ATP was used instead of metaphosphate. The reaction was terminated by immersing the test tube in boiling water for 3 min, and G-6-P formed was assayed by the method of Hohorst⁴².

Determination of inorganic phosphates and acid-labile inorganic phosphates Inorganic phosphates were determined by the method of Fiske and Subbarow⁴³. Acid-labile phosphates were estimated by determining the released inorganic phosphates after being treated at 100 °C for 7 min in 1.0 M HCl.

Chemicals Tripolyphosphate, trimeta- and tetrametaphosphates were purchased from Sigma Chemical Co., St. Louis MO. Metaphosphate and polyphosphate were purchased from Katayama Chemical Industries Co., Osaka, Japan. G-6-P dehydrogenase and NADP were obtained from Oriental Yeast Co., Tokyo, Japan. ATP was also obtained from Kojin Co., Tokyo, Japan. The other chemicals were all analytical grade reagents.

RESULTS

Distribution of polyphosphate glucokinase in microorganisms

Screening was carried out to find strains of microorganisms which would produce polyphosphate glucokinase. As shown in Table I, polyphosphate glucokinase activity was found in some strains

TABLE I. DISTRIBUTION OF G-6-P FORMING ACTIVITY IN MICROORGANISMS

Strain	Activity ^a	
	Phosphoryl donor	
	Metaphosphate	ATP
<i>Acetobacter xylinum</i>	7.0	48.6
<i>Achromobacter butyri</i>	76.2	68.3
<i>Aerobacter aerogenes</i>	3.1	52.9
<i>Alcaligenes faecalis</i>	5.2	59.6
<i>Bacillus natto</i> (SN)	0	66.1
<i>Bacillus subtilis</i>	0	— ^b
<i>Bacterium cadaveris</i>	0	—
<i>Brevibacterium ammoniagenes</i>	3.1	57.2
<i>Brevibacterium flavum</i>	2.2	54.3
<i>Corynebacterium sepeidonicum</i>	0	43.4
<i>Flavobacterium arborescens</i>	0	—
<i>Escherichia coli</i>	0	43.1
<i>Escherichia coli</i> Crookes	0	46.4
<i>Escherichia coli</i> K-12	0	53.2
<i>Escherichia freundii</i>	0	56.6
<i>Micrococcus flavus</i>	63.4	49.1
<i>Micrococcus lysodeikticus</i>	35.2	27.9
<i>Micrococcus glutamicum</i>	4.1	53.3
<i>Proteus mirabilis</i>	0	28.4
<i>Proteus vulgaris</i>	0	36.8
<i>Pseudomonas aeruginosa</i>	0	46.2
<i>Serratia marcescens</i>	0	50.1
<i>Sarcina aurantiaca</i>	0	56.3

^a nmoles of G-6-P formed/mg protein/min.

^b Not determined.

such as *Micrococcus*, *Brevibacterium* species and *Achromobacter butyri*, *Acetobacter xylinum*, *Alcaligenes faecalis* and *Aerobacter aerogenes* also showed slight activity. On the other hand, ATP-dependent glucokinase activity was found in all microorganisms tested. *A. butyri* OUT 8004 showed the highest polyphosphate glucokinase activity among microorganisms. I chose this microorganism as the source of polyphosphate glucokinase in this study.

Phosphorylation of glucose by cell extract of *A. butyri*

Under the standard assay conditions described in MATERIALS AND METHODS, phosphorylation reaction of glucose proceeded linearly with time and protein concentration up to 50 min and 1.0 mg/ml, respectively. In the absence of phosphoryl donor slight synthesis of G-6-P occurred but not after heating the extract at 100 °C for 3 min. Also little synthesis of G-6-P was observed in the absence of Mg²⁺ (Table II). From these data, it was clear that metaphosphate was the sole donor source of phosphorus.

TABLE II. COMPONENT REQUIREMENT FOR G-6-P FORMATION BY CELL FREE EXTRACTS OF *A. butyri*

Activity was determined under standard assay conditions described in METHODS AND MATERIALS.

System	Activity ^a	
	Polyphosphate glucokinase	ATP-dependent glucokinase
Complete	76.9	67.4
-Phosphate donor		
Metaphosphate	0	—
ATP	—	0
-MgCl ₂	0.10	0.09
Boiled extracts	0	0

^a nmoles of G-6-P formed/mg-protein/min.

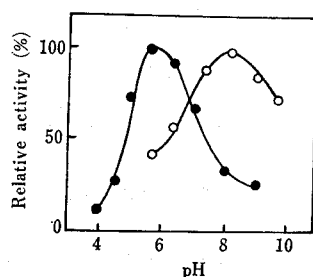


FIG. 1. Effect of pH on Polyphosphate Glucokinase and ATP-dependent Glucokinase Activities.

Activities of the two glucokinase were assayed under standard conditions described in METHODS AND MATERIALS, except for pH. Buffers used were sodium acetate buffer for pH 4.0 to 6.0 and Tris-maleate buffer for pH 5.0 to 9.6. ●, polyphosphate glucokinase; ○, ATP-dependent glucokinase.

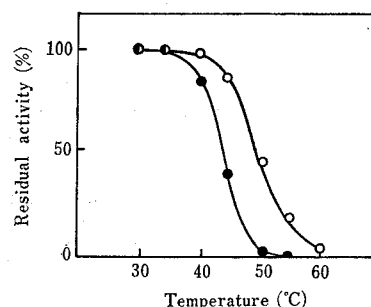


FIG. 2. Thermal Stability of Polyphosphate Glucokinase and ATP-dependent Glucokinase.

The enzymes were treated in Tris-HCl buffer (5 mM, pH 7.0) containing 5 mM $MgCl_2$ for 15 min at the indicated temperature. Residual activities were determined under standard assay conditions. ●, polyphosphate glucokinase; ○, ATP-dependent glucokinase.

Effect of pH on two glucokinase activities

Figure 1 shows the pH-activity profiles for polyphosphate glucokinase and ATP-dependent glucokinase in cell free extracts of *A. butyri*. Polyphosphate glucokinase showed maximum activity at pH 5.5, while ATP-dependent glucokinase had optimum pH at 8.5.

Effect of temperature on stability of two glucokinases in A. butyri

Cell free extracts of *A. butyri* were treated for 15 min at the indicated temperature in Figure 2 and the residual activities were assayed. Polyphosphate glucokinase was extremely unstable in different temperatures. It lost about 60 % of original activity by treatment at 45 °C and was completely inactivated after treatment at 50 °C. On the other hand, ATP-dependent glucokinase retained 50 % of activity even after treatment at 50 °C for 15 min.

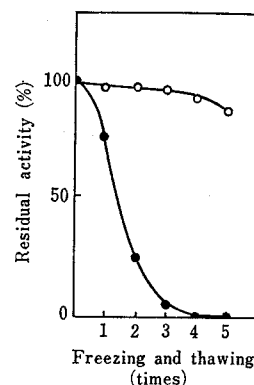


FIG. 3. Effect of Freezing and Thawing on Polyphosphate Glucokinase and ATP-dependent Glucokinase Activities.

The enzyme solutions buffered with 5 mM Tris-HCl buffer (pH 7.0) were frozen and thawed several times, and the residual activities of the two glucokinases were assayed under standard conditions. ●, polyphosphate glucokinase; ○, ATP-dependent glucokinase.

Effect of freezing and Thawing on two glucokinase activities

Cell free extracts of *A. butyri* were freezed and thawed several times, and the changes in activity were examined periodically. As shown in Figure 3, polyphosphate glucokinase was rapidly inactivated by freezing and thawing, whilst ATP-dependent glucokinase was more stable. These results indicate that polyphosphate glucokinase and ATP-dependent glucokinase are different enzymes.

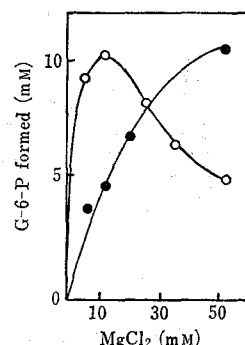


FIG. 4. Effect of Mg^{2+} Concentration on Polyphosphate Glucokinase and ATP-dependent Glucokinase Activities.

Activities were determined under various concentrations of Mg^{2+} . Other conditions were the same as those described in METHODS AND MATERIALS. ●, polyphosphate glucokinase; O, ATP-dependent glucokinase.

Effect of metal ions on polyphosphate glucokinase activity in A. butyri

As shown in Table II, Mg^{2+} was indispensable for appearance of activity. Effects of other metal ions were also examined. Of the metal ions tested, Mg^{2+} was the most effective activator and its effect increased with the concentration up to 60 mM (Fig. 4). Other metal ions, such as Mn^{2+} , Co^{2+} , Ni^{2+} , Ca^{2+} and Cu^{2+}

TABLE III. PHOSPHORYL DONOR SPECIFICITY OF POLYPHOSPHATE GLUCOKINASE

Activity was determined under standard assay conditions described in METHODS AND MATERIALS, except that various phosphate donors were used.

Phosphoryl donor	Concentration	Activity ^a
Pyrophosphate	5 mM	0
Triphosphate	5 mM	0
Polyphosphate	2%	0
Trimetaphosphate	5 mM	0
Tetrametaphosphate	5 mM	0
Metaphosphate	2%	82.1
p-Nitrophenylphosphate	5 mM	0

^a nmoles of G-6-P formed/mg protein/min.

were effective to a lesser extent. But Zn^{2+} , Fe^{2+} and Hg^{2+} were rather inhibitory to polyphosphate glucokinase, though the data are not shown.

Phosphoryl donor specificity of polyphosphate glucokinase in A. butyri

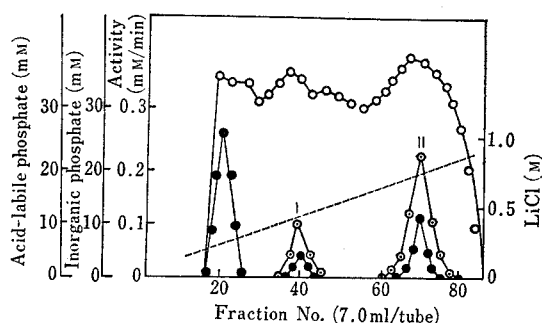


FIG. 5. Elution Pattern of Phosphoryl Donors in Metaphosphate on Dowex 1x2 Column.

Thirty milliliter of 10% aqueous solution of metaphosphate (pH 5.5) was dialyzed against 1.5 liter of water overnight under stirring. Outer solution was passed through the Dowex 1x2 (Cl^- , 200~400 mesh) column (1.2×20 cm) and adsorbed phosphate polymers were eluted with a linear gradient of LiCl from 0 to 1.0 M (pH 2.0). \odot , polyphosphate glucokinase activity; \bullet , inorganic phosphate; \circ , acid labile phosphate; —, concentration of LiCl.

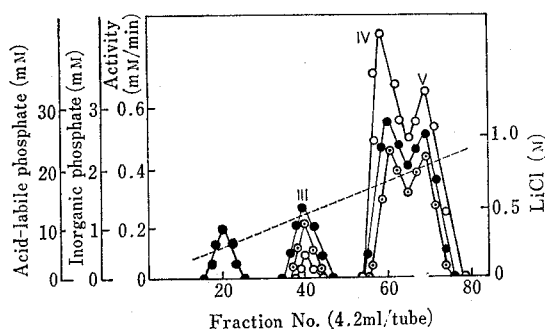


FIG. 6. Elution Pattern of Phosphoryl Donors on Dowex 1x2 Column.

The active fraction eluted with 0.7 M of LiCl on first Dowex column (Fig. 5) was again fractionated on the same column. Conditions were the same as those in Fig. 5. \odot , polyphosphate glucokinase activity; \bullet , inorganic phosphate; \circ , acid labile phosphate; —, concentration of LiCl.

As shown in Table III, metaphosphate was the only phosphoryl-donor, and the other ring phosphate polymers, such as tri- and tetra-metaphosphates were inert. Being independent of the degree of condensation, the chain phosphate polymers were completely inert. Therefore, great interest is attached to the intrinsic phosphoryl donors in metaphosphate, which is a mixture of various ring phosphate polymers.

When metaphosphate solution was dialyzed against water using cellulose tube, about 87 % of phosphoryl donor activity was found in outer solution. This result indicates that the molecular weight of intrinsic phosphoryl donors is below 6×10^3 . The outer solution was passed through Dowex 1 x 2 column, and the adsorbed phosphate polymers were eluted with

a linear gradient of LiCl. As shown in Figure 5, active fractions were observed at two peaks (I and II), which were eluted at 0.4 M and 0.7 M LiCl, respectively. Peak II fractions were pooled, precipitated with cold mixture of acetone-methanol (1:1

v/v) and washed thoroughly with this solvent. Figure 6 shows the rechromatography of peak II on the same column. Active fractions were found at three peaks (III, IV and V). Peak III and IV, which were eluted at 0.4 M and 0.6 M LiCl respectively, seemed to be the degradation products of peak II of Fig. 5.

DISCUSSION

In order to select suitable microorganisms for production of G-6-P, the distribution of polyphosphate glucokinase activity was examined. The distribution of polyphosphate glucokinase activity was observed in a few genera, whereas ATP-dependent glucokinase activity was detected in all microorganisms tested. Furthermore, the distribution of polyphosphate glucokinase activity was closely similar to that of metaphosphate-dependent NAD kinase as described in the section of 1-1 in Chapter I. These facts offer some suggestions for classification of microorganisms and also indicate a characteristic metabolism of phosphorus in microorganisms utilizing metaphosphate.

Explanations for the existence of these microorganisms utilizing metaphosphate may be given in two different ways. One of them is that these microorganisms are considered to be at a lower stage of evolution, since such phosphate polymers are thought to be a vestige of primitive donor of phosphorus and energy⁴⁴⁾. For example, in recent studies on polyphosphate, Kulaev et al.^{45,46,47)} indicated that the inorganic phosphate polymers could perform ubiquitous functions of ATP in microorganisms. Another explanation is that the reaction systems related to phosphate polymers have

been maintained in certain microorganisms, since these phosphate polymers have properties to keep cells in suitable growth surroundings. In fact, phosphate polymers have strong buffer action and minimize disturbance of osmotic equilibrium⁴⁸⁾. Phosphate polymers also act as a chelator of divalent metal ions⁴⁸⁾. Therefore, studies on phosphate polymers are important for elucidation not only of microorganism evolution but also of mechanism of phosphorus metabolism in microorganisms utilizing phosphate polymers.

The enzymatic properties of polyphosphate glucokinase in *A. butyri* were examined. This enzyme was different from ATP-dependent glucokinase in many points. For example, optimal pH and stability to freezing and thawing were clearly different in each. But these two glucokinases in *A. butyri* were not separated by ammonium sulfate fractionation and by DEAE-cellulose column chromatography though the data are not shown. Szymona and Ostrowski⁴⁰⁾ also did not succeed in separating the two glucokinases in *Mycobacterium phlei*. Therefore, the behavior of the two glucokinases in purification processes seemed to be quite alike, though the enzymatic properties of the two glucokinases were entirely different from each other.

In order to examine the intrinsic phosphoryl donors of polyphosphate glucokinase, chromatographic analyses of metaphosphate were carried out. I found at least three substrates in metaphosphate. But it was difficult to separate and obtain these substrates in pure state, since these substrates were very unstable.

On chromatographies, the active fractions corresponded to fractions stained by the Fiske and Subbarow method. This result suggests that phosphoryl donors for polyphosphate glucokinase are ring phosphate polymers, because authentic tri- and tetrametaphos-

phates were also stained by the Fiske and Subbarow method, whereas pyrophosphate, tripolyphosphate and highly condensed chain phosphate polymers were not.

Thus, the results obtained indicated that *A. butyri* contains two different types of glucokinase, ATP-dependent glucokinase and polyphosphate glucokinase. The application of polyphosphate glucokinase to the continuous production of G-6-P is shown in next section.

SUMMARY

The activities of polyphosphate glucokinase which synthesize G-6-P from glucose and metaphosphate were found in some microorganisms. This enzyme occurred most abundantly in *Micrococcus* and *Achromobacter* species and less in *Brevibacterium*, *Aerobacter* and *Alcaligenes species*. The distribution pattern of this enzyme was closely similar to that of metaphosphate-dependent NAD kinase. Polyphosphate glucokinase in *A. butyri* was different from ATP-dependent glucokinase in some enzymatic properties. The potent phosphoryl donor for this enzyme was metaphosphate, and other chain or ring phosphate polymers were not utilized by this enzyme. Metaphosphate, a mixture of various ring phosphate polymers, contained at least three intrinsic phosphoryl donors for polyphosphate glucokinase.

2-2 Continuous production of G-6-P by immobilized *Achromobacter butyri* cells

INTRODUCTION

In previous section 2-1, the distribution and properties of polyphosphate glucokinase activity (EC 2.7.1.a) were studied and this enzyme was shown to be suitable for the production of G-6-P. Following these investigations, an efficient continuous method for the production of G-6-P from glucose and metaphosphate using immobilized *Achromobacter butyri* cells was studied.

MATERIALS AND METHODS

Cultivation, immobilization and activation of microorganisms

Microorganisms were cultivated at 30 °C for 20 h in the Medium A described in section 1-1 in Chapter I and immobilized as described by Chibata et al.^{3,4}, in which 0.3 g (wet wt) of intact cells was immobilized in 1.0 ml of polyacrylamide gel. The immobilized cells (10 ml) were transferred to 200 ml of acetone and incubated at 25 °C for 30 min with stirring. Intact cells (3.0 g) were also suspended in 200 ml of acetone and incubated in the same manner.

Determination of polyphosphate glucokinase activity Unless otherwise given, the following reaction system was used for the determination of polyphosphate glucokinase activity. A reaction mixture containing 500 µmol glucose, 0.2 g metaphosphate, 200 µmol MgCl₂, 500 µmol Tris-HCl buffer (pH 7.0) and 2 ml immobilized cells or 0.6 g intact cells (volume 10 ml) was incubated with shaking at 37 °C for 10 min. The raw extract from intact cells and the homogenate of immobilized cells were also used as enzyme sources. The raw extract from intact cells was prepared as follows: 1.0 g intact cells was suspended in 3.0 ml of 5.0 mM Tris-HCl buffer (pH 7.0) and subjected to ultrasonic treatment at 9 KHz for 10 min. An immobilized cell homogenate was prepared by grinding 3.0 ml of gel with 5.0 ml of 5.0 mM Tris-HCl buffer (pH 7.0) at 0 °C for 20 min. G-6-P formed in reaction mixture was determined by the method of Hohorst⁴². Activity was expressed as µmoles of G-6-P formed per g of intact cells per min.

Determination of inorganic phosphates and acid-labile inorganic phosphates The methods for the determination of inorganic phosphates and acid-labile inorganic phosphates were the same as described in section 2-1.

RESULTS

Properties of immobilized cells

1) *Activation of immobilized cells* In general, it was necessary to remove the cell membrane barrier in order to enable efficient transport of phosphate esters into cells, and so immobilized cells were treated

Table 1. Activation of immobilized *A. butyri* cells

Treatment with	Activity ^a after treatment for	
	30 min	60 min
0.85 % NaCl	0.1	0.2
10 % Ethanol	0.2	1.1
10 % Toluol	2.4	4.2
100 % Acetone	6.9	8.4
0.1 % Cation-S	0.7	1.4
0.1 % SDS	0.6	1.6

^a $\mu\text{mol/g-cells/min}$

Immobilized cells (10 g) were suspended in 200 ml of various solutions and incubated at 25°C for 30-60 min with stirring. The polyphosphate glucokinase activity after treatment was assayed under standard conditions as described in Materials and Methods

with inorganic solvents or detergents. Acetone was most effective for activation of polyphosphate glucokinase activity (Table 1) and was routinely employed to activate immobilized cells. Intact cells were also activated in the same manner.

2) Polyphosphate glucokinase activity of immobilized microbial cells

To select the most active immobilized microorganisms, several microorganisms having polyphosphate glucokinase activity were immobilized in polyacrylamide gel lattice and enzyme activities were determined. As shown in Table 2, the immobilized *A. butyri* cells showed the highest polyphosphate glucokinase activity among the strains tested. A summary of polyphosphate glucokinase activity of *A. butyri* cells after acetone treatment is shown in Figure 1. The intrinsic activity in cells

Table 2. Polyphosphate glucokinase activity of immobilized microbial cells

Strain	Activity ^a	
	Acetone-treated cells	Acetone-treated immobilized cells
<i>Achromobacter butyri</i>	7.5	8.4
<i>Acetobacter xylinum</i>	0.8	0.6
<i>Micrococcus flavus</i>	6.5	6.3
<i>Micrococcus lysodeikticus</i>	3.8	3.6

^a $\mu\text{mol/g-cells/min}$

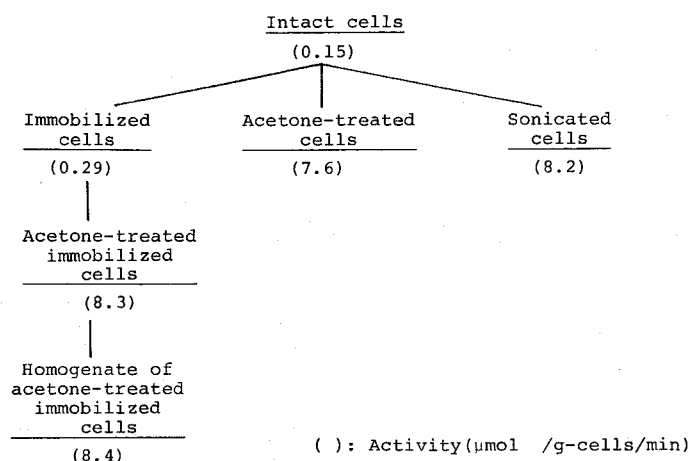


Fig. 1. Comparison of polyphosphate glucokinase activities in cells after various treatments

estimated by using a raw extract was $8.2 \mu\text{mol/g-cells/min}$ and that of the acetone-treated immobilized cells was $8.3 \mu\text{mol/g-cells/min}$. Thus, polyphosphate glucokinase was immobilized in polyacrylamide gel without an activity loss. Since the activities were nearly equal, the polyacrylamide gel matrix was thought not to be a barrier for the transport of substrates and/or products and *A. butyri*, thus chosen as suitable microorganism for G-6-P production, was used in further studies. In the following

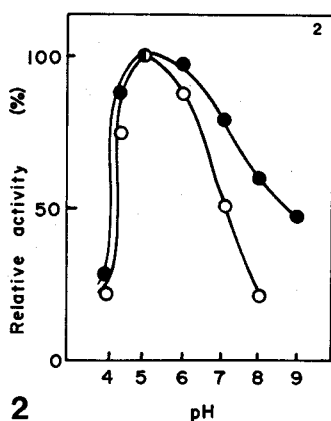


Fig. 2. Effect of pH on G-6-P formation by intact cells and immobilized cells. The pH was adjusted to indicated values with 0.1 M Tris-maleate buffer. Other conditions were the same as described in Materials and Methods. Symbols: (○), intact cells; (●), immobilized cells

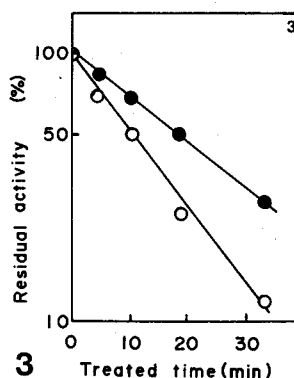


Fig. 3. Thermal stability of polyphosphate glucokinase of intact cells and immobilized cells. The intact and immobilized cells were suspended in 5.0 mM Tris-HCl buffer (pH 7.0) containing 5.0 mM MgCl_2 and incubated at 50°C under the conditions described in Materials and Methods. Symbols: (○), intact cells; (●), immobilized cells

experiments, unless otherwise given, intact cells and immobilized cells represent the acetone-treated intact cells and acetone-treated immobilized cells, respectively.

3) Effect of pH on polyphosphate glucokinase activity of immobilized *A. butyri* cells

Figure 2 shows the pH profile of polyphosphate glucokinase activity of immobilized cells and intact cells. The pattern of pH dependence on the rate of G-6-P formation was slightly changed in the alkaline region by immobilization. The optimal pH was 5.0 for both preparations.

4) Stability of polyphosphate glucokinase of immobilized *A. butyri* cells

Comparing the thermal stability of polyphosphate glucokinase of immobilized cells with that of intact cells, the thermal stability of immobilized cells was greater at higher temperature (Fig. 3). The operational stability of polyphosphate glucokinase of both

preparations was also examined through repeated use (Table 3). Although, intact cells rapidly lost enzyme activity, that of the immobilized cells was stable and did not decrease after repeated use.

Production of G-6-P by immobilized *A. butyri* cells

1) pH effect

Figure 4 shows the profile of G-6-P pro-

Table 3. Reuse of immobilized cells and intact cells

Reuse (No. of times)	Polyphosphate glucokinase activity ^a	
	Intact cells	Immobilized cells
1	100	100
2	73	106
3	40	96
4	30	93
5	25	95

^a Relative activity

Enzyme activity was determined under the standard conditions described in Materials and Methods except that the reaction was carried out for 60 min. The intact cells and immobilized cells were thoroughly washed with 5.0 mM Tris-HCl buffer (pH 7.0) each time before the addition of a new substrate solution

Fig. 4. Effect of pH on G-6-P production by immobilized cells. Immobilized cells (0.3 g) were incubated in a reaction mixture containing 0.1 M glucose, 10 mM MgCl_2 , and 100 mg/ml metaphosphate for 20 h at 37°C with shaking. Metaphosphate was added after adjustment to the indicated pH values

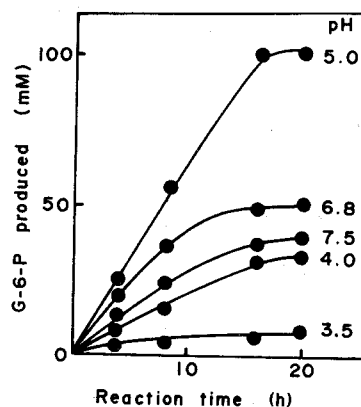
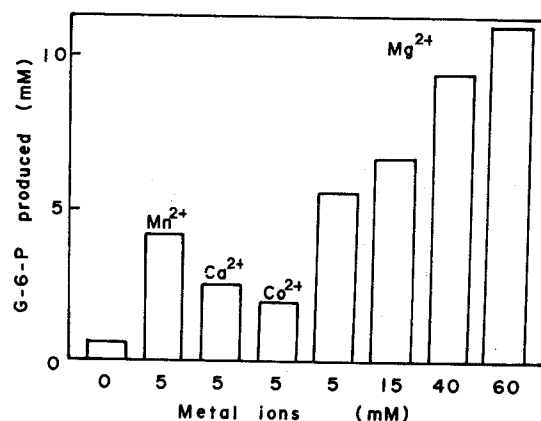


Fig. 5. Effect of metal ions on G-6-P production by immobilized cells. Activity was determined under standard assay conditions as described in Materials and Methods, except that various metal ions were used at the indicated concentrations



duction at various pH values. G-6-P was effectively produced at pH 5.0, the optimum pH of polyphosphate glucokinase. During G-6-P production, pH of reaction mixture was kept constant by the bufferine action of metaphosphate.

2) *Metal ion effect* Divalent metal ions were required for the production of G-6-P as shown in the section 2-1 in Chapter 2. Of the metal ions tested, Mg^{2+} was the most effective activator. Mn^{2+} , Co^{2+} and Ca^{2+} also activated the polyphosphate glucokinase reaction (Fig. 5). The production rate of G-6-P increased with increasing concentration of Mg^{2+} up to 60 mM.

3) *Effect of metaphosphate concentration* Production of G-6-P was investigated at various metaphosphate concentration.

As shown in Figure 6, the amount produced was proportional to the metaphosphate concentration. Under the investigated conditions, 1.0 mg of metaphosphate was required to completely convert 1.0 μmol of glucose to G-6-P. One mg of metaphosphate contained approximately 25 μmole of acid-labile phosphate. The chemical structure of metaphosphate, however, was unclear, since metaphosphate was a mixture of various ring phosphate polymers. Hence, the stoichiometric correlation between glucose and metaphosphate in the phosphorylation of glucose was difficult to assess.

4) Continuous production of G-6-P with immobilized cell column

Figure 7 shows the relationship between the flow rates and amount of G-6-P formed. At the flow rates below $\text{S.V.} = 0.5 \text{ h}^{-1}$, 0.1 M glucose was almost completely converted to G-6-P. Figure 8

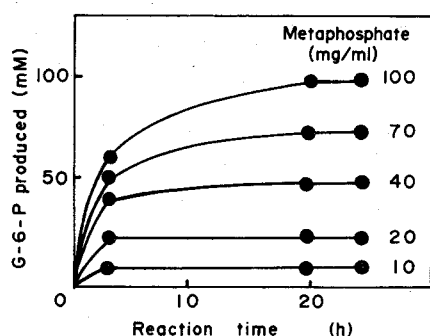


Fig. 6. Effect of metaphosphate concentration on G-6-P production by immobilized cells. The reaction was carried out in the presence of 0.1 M glucose. Other conditions were the same as described in Materials and Methods except for metaphosphate concentrations

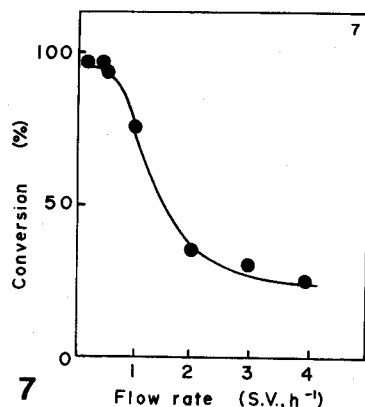


Fig. 7. Effect of flow rate on G-6-P production by immobilized cell column. Immobilized cells (5.0 g) were packed in column (1.0 x 10 cm) and substrate solution (37°C) containing 0.1 M glucose, 40 mM MgCl_2 , and 100 mg/ml metaphosphate (pH 5.0) was passed upward through the column at the various flow rates. The activity of the column was checked by determining G-6-P present in effluent

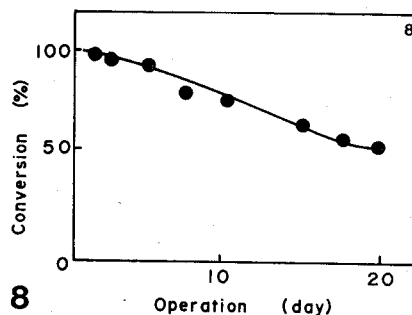


Fig. 8. Stability of immobilized cell column. Substrate solution described in Figure 7 was continuously passed through the column at $\text{S.V.} = 0.26 \text{ h}^{-1}$. Other conditions were the same as described in Figure 7

shows the column deterioration at the flow rate $S.V.=0.26\text{ h}^{-1}$. The column half-life was approximately 20 days. On the other hand, the half-life of the intact cells, calculated from the data in Table 3, was approximately 3 h.

DISCUSSION

The continuous production of G-6-P was investigated using a column packed with acetone-treated immobilized *A. butyri* cells. As a result, G-6-P was continuously produced by this column in a high yield. A similar method starting with p-nitrophenyl phosphate and glucose was described by Saif et al.³⁹⁾, who used immobilized cells of *Escherichia freundii* K-1 having phosphotransferase activity. Their method was not always satisfactory, however, since G-1-P was also produced in relatively high ratios. The present method has the following advantages: (1) the only reaction product is G-6-P, and (2) G-6-P is produced economically since the metaphosphate is available at a much lower price than p-nitrophenyl phosphate. The results also indicate that phosphate polymers are utilized by cells immobilized in polyacrylamide gel lattice. Therefore, continuous production of useful materials is expected to be effectively carried out with immobilized cells which have enzymes utilizing phosphate polymers.

SUMMARY

Whole cells of *Achromobacter butyri* OUT 8004 having polyphosphate glucokinase activity were immobilized in polyacrylamide gel lattice. The immobilized cells were activated by organic solvents, especially acetone. The immobilization resulted in increased stability of polyphosphate glucokinase. Continu-

ous high yield production of G-6-P from glucose and metaphosphate was carried out with an immobilized cell column, which had a half-life of approximately 20 days.

CAPTER II

Application of glycolytic pathway in yeast cells as ATP regeneration system for production of glutathione

Section 1 Glutathione production by immobilized *Saccharomyces cerevisiae* cells

INTRODUCTION

Many enzyme processes, especially multienzyme systems, require the participation of coenzymes and/or a supply of energy (ATP). Therefore, the construction of regeneration systems coenzymes and/or ATP is indispensable for the economic utilization of these enzyme reactor systems. From this standpoint, Langer et al.²⁾ evaluated the relative routes to ATP regeneration involving chemical synthesis, whole cells or organelles, or cell free enzymes. Among these routes to ATP regeneration, I thought that the utilization of microbial whole cells is more advantageous, since it excludes the intricate and tedious procedures for the preparation of organelles or enzymes.

The basis for the utilization of whole cells as an ATP regeneration was demonstrated by Tochikura and his groups. They studied the utilization of the glycolytic pathway in yeast as an ATP regeneration system³⁾, and they succeeded in applying this system to the production of useful materials such as cytidine diphosphate choline⁴⁾, and 6-phosphogluconate⁵⁾ by batch process. From the industrial standpoint, however, a continuous reaction system using immobilized cells was considered to be more advantageous than a batch process.

Thus, in order to develop a more efficient method, I investi-

gated the usefulness of the glycolytic pathway in yeast as an ATP regeneration system in a reactor for glutathione production.

MATERIALS AND METHODS

Chemicals NAD was purchased from Oriental Yeast Co., Ltd., Tokyo, Japa

Cultivation and immobilization of Saccharomyces cerevisiae cells *S. cerevisiae* IFO 2044 was cultivated in 100 ml of medium containing 0.5 % glucose, 1.0 % peptone, 1.0 % yeast extract, 0.5 % meat extract and 0.5 % NaCl (pH 6.0) at 30 °C for 20 h with reciprocal shaking. After cooling the broth to 0 °C, cells were harvested by centrifugation, and resuspended in 0.85 % cold saline solution. The cell suspension was centrifuged at 10,400 xg for 10 min, and weighed. For immobilization in polyacrylamide gel, harvested cells (28 g wet wt) were suspended in 15 ml of 30 % KCl solution. To this cell suspension, 15 ml of 33.5 % acrylamide monomer solution containing 2.0 % N,N'-methylenebisacrylamide, 5.0 ml of 5.0 % β -dimethylamino-propionitril and 6.0 ml of 6.5 % potassium persulfate were successively added at 0 °C. The mixture was slowly warmed to 20 °C and allowed to stand until a gel was formed. The resulting stiff gel was cut into cubic (2 x 2 x 2 mm) granule and washed with saline solution. By this method, 80 ml of gel was obtained containing 0.35 g (wet wt) cells per ml of gel.

Assay of glutathione-producing activity Immobilized cells (8 ml) or intact cells (2.8 g wet wt) were incubated in 20 ml of a mixture containing 0.5 M glucose, 0.01 M MgCl₂, 0.02 M L-glutamate, 0.02 M L-cysteine, 0.02 M glycine and 0.1 M potassium phosphate buffer (pH 7.0) at 30 °C for several hours with shaking. At a prescribed time, 0.5 ml of the reaction mixture was removed and the concentration of reactants and products were determined. Glutathione was determined by the method of Tietze⁴⁹. FBP and glucose were determined by the methods of Roe and Papadopoulos⁵⁰, and Hugget and Nixon⁵¹, respectively.

Extraction of glutathione from cells The 0.5 ml sample was heated in a boiling water for 1 min, chilled immediately in ice water and centrifuged to remove cell debris. Using the clear supernatant, glutathione formed by intact cells was determined. Temperature and time for extraction and recovery experiments of glutathione (data not shown) showed that this extraction method was adequate to extract glutathione from the cells.

RESULTS

Reactor system for the production of glutathione

Figure 1 shows the reaction systems for glutathione produc-

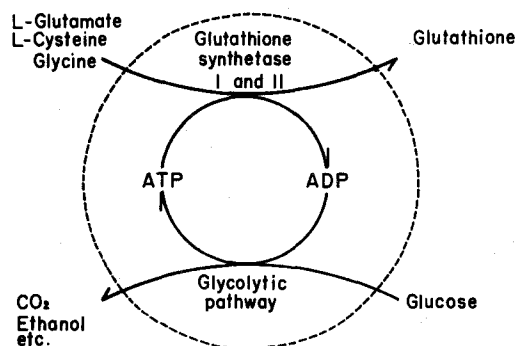


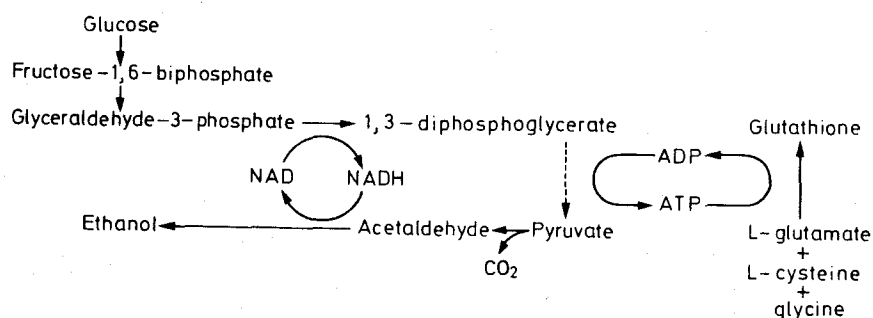
Fig. 1. Schematic representation of reactor system for production of glutathione. Details of scheme are described in the text. Dotted line in figure shows the polyacrylamide gel lattice

tion. This reactor consists of two reaction systems. One is the ATP regeneration system containing the enzymes in the glycolytic pathway. By this system, ATP is converted to ADP, consuming glucose. This system also allows the regeneration of NAD in the course of the assimilation of glucose to ethanol and carbon dioxide (Scheme 1). The other is the γ -

glutamylcysteine synthetase, designated GSH-I, and glutathione synthetase, designated GSH-II, system. These two reaction systems are contained in *Saccharomyces cerevisiae* cells. So the cells were immobilized in polyacrylamide gel lattice following the method of Chibata et al.³⁴⁾.

Glutathione production by intact and immobilized cells

The patterns of glutathione production by intact and im-



Scheme 1. The glycolytic pathway (ATP and NAD regenerating system) and the transformation system

bilized cells are shown in Figure 2. As is well known, glutathione produced by intact cells was accumulated in the cells and was not excreted. To estimate the glutathione-producing activity of intact cells, heat treatment of cells was necessary for glutathione extraction. Not only the accumulation of glutathi-

one, but also extracellular accumulation of FBP, a key metabolite of the glycolytic pathway, was not detected (Fig. 2A). On the other hand, in the case of immobilized cells (Fig. 2B), glutathione produced was excreted into the medium from the gel, and a considerable amount of FBP was also found extracellularly.

Conditions for immobilization

Table 1 shows the effect of culture time on glutathione-

Table 1. Effect of culture time on glutathione-producing activity of intact and immobilized cells

Culture time (h)	Growth (mg of cells/ml of broth)	Glutathione-producing activity ^a of	
		Intact cells	Immobilized cells
16	15.5	1.97	2.26 (0.80) ^b
24	18.4	2.12	2.40 (0.85)
40	17.3	1.64	1.84 (0.65)
64	15.4	0.99	1.11 (0.39)

^a μ mole of glutathione produced/g (wet wt.)-cells/h

^b μ mole of glutathione produced/ml-gel/h

S. cerevisiae cells were grown on the medium described in Materials and Methods for several hours. In case of intact cells, activity was determined by extracting glutathione from the cells as described in Materials and Methods

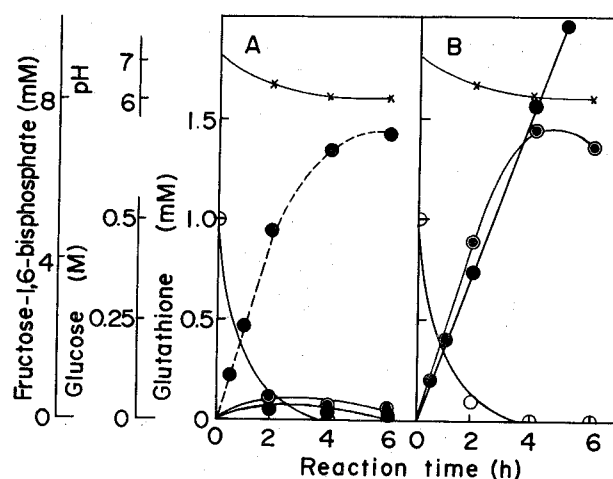


Fig. 2A and B. Glutathione production by intact cells (A) and immobilized cells (B). Reaction conditions were the same as described in Materials and Methods. In case of intact cells, glutathione in cells (---●---) and in the extracellular medium (—●—) were determined. Symbols: (●), glutathione; (○), glucose; (◐), fructose-1,6-bisphosphate; (x), pH

producing activity of intact cells and immobilized cells. The most favorable glutathione-producing activity was obtained by immobilizing cells grown for 24 h.

For the immobilization of cells grown for 24 h, the optimum acrylamide monomer

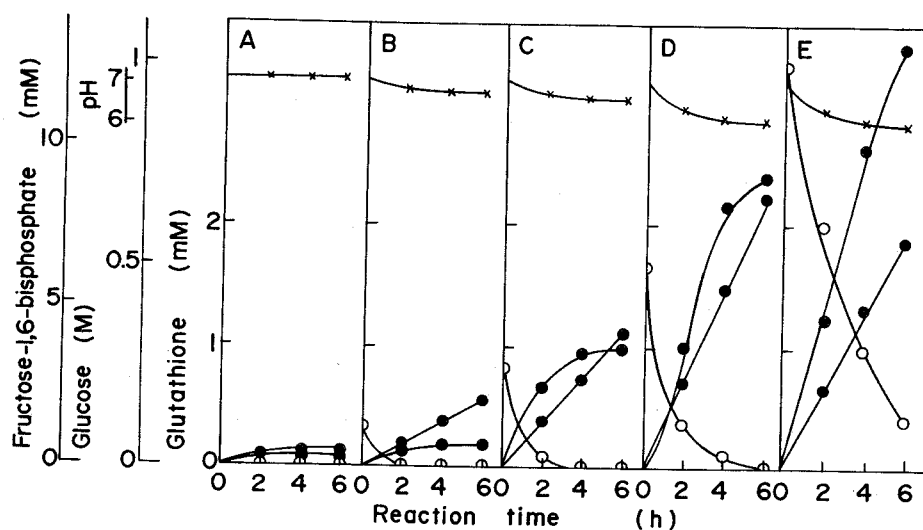


Fig. 3
Effect of glucose concentration on glutathione production by immobilized cells. Reaction was carried out for several hours as described in Materials and Methods. Glucose concentrations were: 0 M (A); 0.13 M (B); 0.25 M (C); 0.5 M (D) and 1.0 M (E). Symbols: (●), glutathione; (○), glucose; (◐), fructose-1,6-bisphosphate; (x), pH

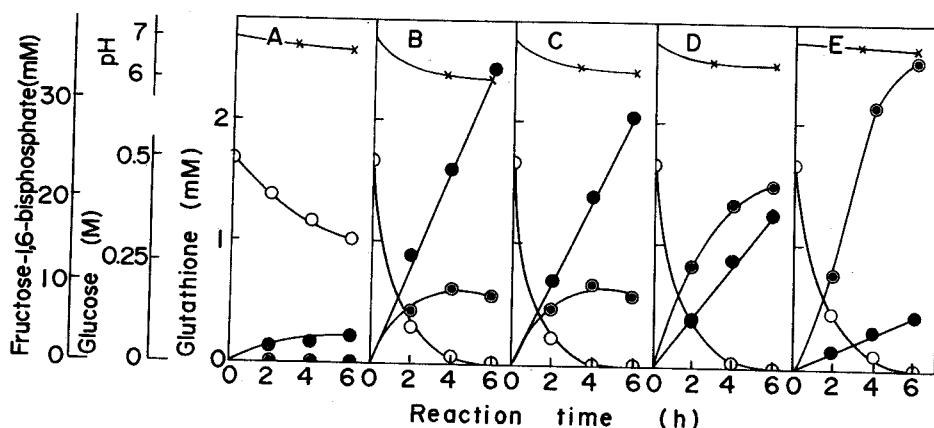


Fig. 4.
Effect of phosphate buffer concentration on glutathione production by immobilized cells. Reaction was carried out for several hours as described in Materials and Methods. Phosphate buffer concentrations used were: 0 M (A); 0.05 M (B); 0.1 M (C); 0.2 M (D); and 0.5 M (E). Symbols: (●), glutathione; (○), glucose; (◐), fructose-1,6-bisphosphate; (x), pH

concentration was found to be 80 mg/ml. At concentrations above 80 mg/ml, the monomer reduced the glutathione-producing activity of the immobilized cells. Thus, the monomer concentration for immobilization was 80 mg/ml throughout this study.

Conditions of glutathione production by immobilized cells

As Figure 2B shows, the immobilized cells produced glutathione from the constituent amino acids while consuming glucose, and the glutathione produced was excreted into the medium. I next investigated the optimum conditions for glutathione production.

1) Effect of glucose concentration

In my glutathione production system, glucose is essential to generate ATP; thus glucose concentration may influence the glutathione-producing activity of immobilized cells. Figure 3 shows the time course of glutathione production with various glucose concentration. In the absence of glucose (A), little glutathione was produced. Glutathione production was increased with glucose concentration up to 0.5 M. Similarly, FBP accumulation also increased according to the glucose concentration.

2) Effect of phosphate concentration

The effect of phosphate buffer concentration on glutathione production is shown in Figure 4. Glutathione production was efficient at low concentration of phosphate buffer, but was reduced in the absence of phosphate buffer (A). On the other hand, accumulation of FBP increased with phosphate concentration.

3) Effect of $MgCl_2$ concentration

The effect of $MgCl_2$ concentration on glutathione-producing activity was investigated. The optimum $MgCl_2$ concent-

Table 2. Amino acid requirement for glutathione production with immobilized cells

Amino acid omitted	Glutathione-producing activity ^a
None	2.45 (0.87) ^b
L-glutamate	1.68 (0.58)
L-cysteine	0 (0)
Glycine	1.44 (0.41)
L-glutamate and glycine	0.96 (0.32)

^a μ mole of glutathione produced/g (wet wt.)-cells/h

^b μ mole of glutathione produced/ml-gel/h

Glutathione-producing activity was assayed under standard conditions as described in Materials and Methods with or without amino acid omissions

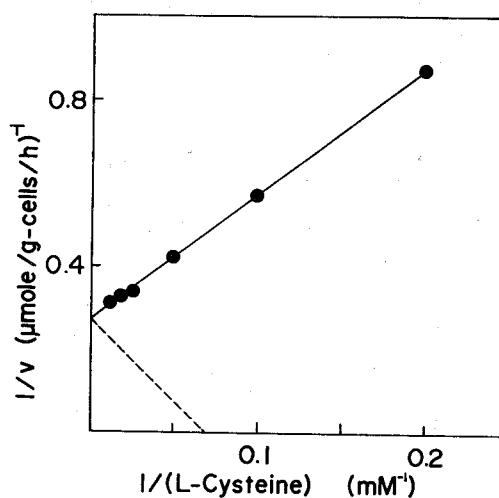


Fig. 5. Effect of varying concentration of L-cysteine on glutathione production by immobilized cells. Reaction was carried out under the same conditions as described in Materials and Methods with the exception of L-cysteine concentration. The data are presented as a double reciprocal plot of the rate of glutathione production as a function of L-cysteine concentration

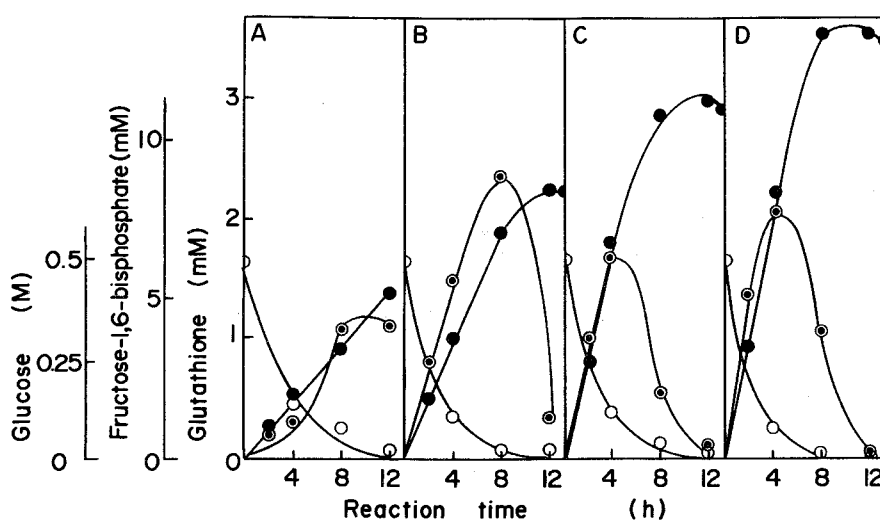


Fig. 6. A-D
Time course of glutathione production with various amounts of immobilized cells. Reaction was carried out in 20 ml of mixture containing 0.5 M glucose, 0.02 M MgCl_2 , 0.025 M L-glutamate, 0.025 M L-cysteine, 0.025 M glycine, 0.05 M potassium phosphate buffer (pH 7.0) and 2.0 ml (A), 4.0 ml (B), 6.0 ml (C) or 8.0 ml (D) of immobilized cells. Other reaction conditions were the same as described in Materials and Methods. Symbols: (●), glutathione; (○), glucose; (⊙), fructose-1,6-bisphosphate

ration was found to be 0.02 M.

4) Effect of initial pH

As shown in Figure 2, the pH of the reaction mixture decreased with increased glucose consumption; about one pH unit shift was observed for each 0.5 M glucose used. Hence, the effect of initial pH on glutathione production was investigated. The initial pH 6.5 to 7.0 seems to be favorable for glutathione production. Starting the reaction in this pH range, the pH of reaction mixture decreased to about 6.0 after complete consumption of 0.5 M glucose.

5) Effect of amino acids concentrations

Table 2 shows the amino acid requirement for glutathione production. Although the glutathione-producing activity was considerably lowered in the absence of L-glutamate and glycine, these amino acids were not necessarily required for glutathione production by freshly prepared immobilized cells. This is presumably due to the L-glutamate or glycine carried over in the assay mixture by *S. cerevisiae* cells. On the other hand, L-cys-

ties of intact cells and immobilized cells under optimal conditions. The glutathione-producing activity of immobilized cells was higher than that of intact cells, as was FBP accumulation.

7) Reuse of immobilized *S. cerevisiae* cells for glutathione production

The operational stability of glutathione-producing activity of immobilized *S. cerevisiae* cells was examined by repeating the reaction in batches. The gel used once for reaction almost completely

Table 4. Reuse of immobilized *S. cerevisiae* cells for glutathione production

Reuse (No. of times)	Addition to reaction mixture	Glutathione ^a produced (mg/ml/4 h)	Fructose-1,6-biphosphate ^a accumulated (mM/4 h)
1		0.33 (100.0) ^b	8.3 (100.0) ^a
2		0.066 (20.0)	1.3 (15.7)
	10 ⁻⁷ M NAD	0.073 (22.1)	1.6 (19.3)
	10 ⁻⁶ M NAD	0.30 (90.9)	7.4 (89.2)
	5 x 10 ⁻⁵ M NAD	0.35 (106)	8.7 (105)
	5 x 10 ⁻⁵ M NAD ^c	0.01 (3.3)	0.05 (0.6)
	5 x 10 ⁻⁴ M NAD	0.38 (115)	8.9 (107)
	2 x 10 ⁻³ M ATP	0.069 (20.9)	1.2 (14.5)
3		0.061 (18.5)	0.9 (10.8)
	5 x 10 ⁻⁵ M NAD	0.35 (106)	8.6 (104)

^aAmounts of glutathione produced and fructose-1,6-biphosphate determined after the reaction for 4 h with or without the addition of NAD or ATP. Other conditions for reaction are given in the legend to Figure 2. The immobilized cells were thoroughly washed with 0.85% saline solution each time before the addition of a new substrate solution

^bRelative activity

^cNAD was added to the reaction mixture in the absence of glucose

lost its glutathione-producing activity (Table 4). The loss of glutathione-producing activity was thought to be the relative decrease in glycolytic activity, since the loss of glutathione producing activity correlated with the decrease in FBP accumulation (Table 4). So, the effect of addition of ATP and/or NAD was investigated. NAD was proved to be the factor required for the recovery of the glycolytic activity, though the effect of ATP was not observed. NAD at 5 x 10⁻⁵ M was sufficient for the recovery of deteriorated glutathione-producing activity of immobilized cells (Table 4)

Continuous production of glutathione by an immobilized cell column

teine was absolutely required for glutathione production and the Michaelis constant (K_m) for L-cysteine was calculated to be 14.3 mM from the Lineweaver-Burk plot⁵²⁾ (Fig. 5). By considering the K_m value for L-cysteine, the concentration of the three amino acids was set at 0.025 M each, corresponding to twice of K_m for L-cysteine.

Table 3. Glutathione-producing activity and FBP-accumulating ability of intact and immobilized cells

Cell preparation	Glutathione-producing activity ^a	FBP-accumulation at 4 h reaction (mM)
Intact cells	3.16	0.21
Immobilized cells	3.71 (1.31) ^b	10.7

^a μ mole of glutathione produced/g (wet wt.)-cells/h

^b μ mole of glutathione produced/ml-gel/h

Glutathione-producing activity was assayed by incubating the intact cells (2.8 g wet wt.) of immobilized cells (8 ml) in the 20 ml of reaction mixture containing 0.5 M glucose, 0.02 M $MgCl_2$, 0.025 M L-glutamate, 0.025 M L-cysteine, 0.025 M glycine, and 0.05 M potassium phosphate buffer (pH 7.0) at 30 °C with shaking

6) Glutathione production by various amounts of immobilized cells

Based on the data shown above, the optimum concentrations of reactants were determined to be as follows: 0.5 M glucose, 0.05 M potassium phosphate buffer (pH 7.0), 0.02 M $MgCl_2$, 0.025 M for each of the amino acids corresponding to twice that of the K_m for L-cysteine. Figure 6 shows the time course of glutathione production by various amounts of immobilized cells under optimum conditions. The concentration of glutathione initially increased, reached a maximum, and then slowly declined. The glutathione-producing activity was almost proportional to the amount of immobilized cells, and the glutathione-producing activity of immobilized cells calculated from the data was 1.31 μ mole/h/ml-gel.

Table 3 shows the comparison of glutathione-producing activi-

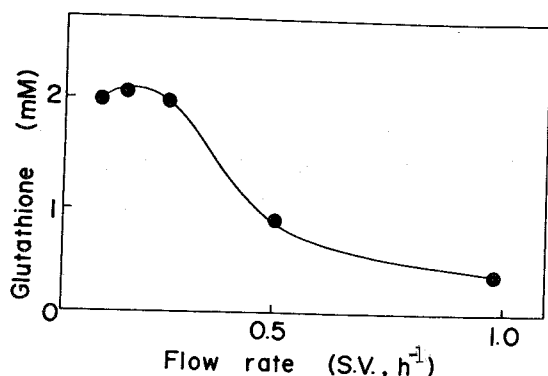


Fig. 6. Effect of flow rates of substrate solution on glutathione production. Ten milliliter of immobilized cells were packed in a column (1.0 cm \times 10 cm) and substrate solution containing 0.5 M glucose, 0.02 M MgCl_2 , 0.025 M L-glutamate, 0.025 M L-cysteine, 0.025 M glycine, 0.05 M potassium phosphate buffer (pH 7.0) and 0.5 mM NAD was passed through this column at various flow rates at 30 °C. Glutathione in the effluent was determined

To produce glutathione continuously, immobilized cells were packed in a column and the substrate solution shown in the legend of Figure 7 was passed upward through this column. Figure 7 shows the effect of flow rates (space velocity, S.V.) of substrate solution on glutathione production. A flow rate of S.V. = 0.15 to 0.20 h^{-1} gave the maximum amount of glutathione in the effluent. From the in-

dustrial standpoint, the useful-life time of glutathione-producing activity is one of the important factors in evaluating the feasibility of the glutathione production system. Therefore, to determine operational stability, the substrate solution was continuously supplied at S.V. = 0.17 h^{-1} at 30 °C. As a result, about 2.0 mM of glutathione was continuously produced by this column, and the half-life of this column was calculated to be 26 days (Figure 8).

To isolate the glutathione, continuous production was carried out for 5 days. The pH of the effluent (about 200 ml containing 131 mg glutathione) was brought to 3.0 with 5.0 M H_2SO_4 and the solution was passed through the column packed with cation exchange resin (Diaion PK-228 H^+). The absorbed glutathione was eluted with 0.5 M NH_4OH and pH of the eluate was adjusted to 4.5 with 5 M H_2SO_4 . The resultant solution was absorbed onto anion

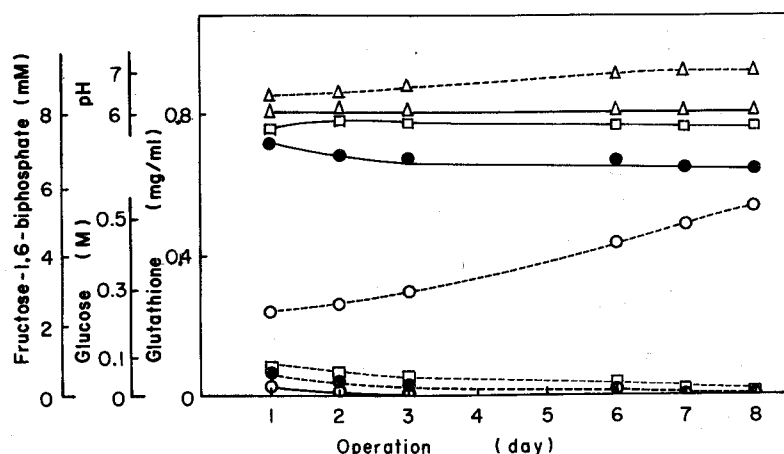


Fig. 8. Continuous production of glutathione by immobilized *S. cerevisiae* cell column. 7.0 g of gel was packed in column (1.2 x 14 cm) and substrate solution was passed through this column at a flow rate of 2.5 ml/h, at 30°C. Substrate solution contained 20 mM *L*-glutamate, 20 mM *L*-cysteine, 20 mM glycine, 10 mM MgCl₂, 0.1 M potassium phosphate buffer (pH 7.0), and 0.5 M glucose, with (—) or without (-----) 5×10^{-4} M NAD. Symbols: (●), glutathione; (○), glucose; (△), pH; (□), fructose-1,6-biphosphate

exchange resin (Duolite A-2, CH₃COO⁻) and eluted with 0.5 M H₂SO₄. The eluate was concentrated and glutathione was crystallized from 50 % ethanol with a yield of 73 %.

DISCUSSION

To produce glutathione efficiently, an ATP supply is indispensable, since this tripeptide is biosynthesized via ATP-requiring reactions catalyzed by γ -glutamylcysteine synthetase and glutathione synthetase. Thus, a glutathione synthetic process was coupled with the glycolytic pathway as an ATP regeneration system, and glutathione was found to be continuously produced by the column packed with immobilized *S. cerevisiae* cells. The characteristics of this glutathione production system may be summarized as follows.

By using the immobilized yeast cells with polyacrylamide gel, glutathione was excreted from the cells. As shown in Fig. 2A, in the case of intact cells, glutathione was accumulated inside the cells and was not excreted out of the cells. Thus, industrial production of glutathione has been mainly carried out by extracting it from cells, although the glutathione content of the cells

is low and extraction procedures are tedious and intricate. Fortunately, however, immobilization of yeast cells with polyacrylamide gel removes the barrier of glutathione transport across the cell membrane. Although the modification caused by immobilization is unknown, this excretion of glutathione is very promising for efficient glutathione production, since extraction from the cells can be eliminated and feedback inhibition of γ -glutamylcysteine synthetase by intracellular glutathione may be lowered.

To produce glutathione, a high glucose concentration was necessary (Fig. 3). If 1 mol of glucose generates 2 mol of ATP in the conversion of glucose to ethanol and carbon dioxide, the utilization efficiency of generated ATP for glutathione production is only 0.5 %, as calculated from the data in Figure 3. To explain the low efficiency of ATP utilization, the following three reasons were considered: (i) ATP generated is degraded by the action of ATPase or phosphatases; (ii) the affinity of glutathione synthetic enzymes for ATP is low in the "immobilized state", or (iii) ATP generated is not fully available to the site of glutathione synthetic reactions. I am now investigating how the efficiency of ATP utilization is influenced by the conditions under which the glutathione synthetic reactions are carried out.

Phosphate buffer inhibits glutathione production. However, glutathione production was markedly reduced in the absence of buffer, although glucose consumption gradually proceeded. This indicates that the rapid consumption of glucose, that is, a highly potent ATP supply, was necessary for glutathione production. As mentioned above, to enhance ATP generation over ATP degradation and to compensate for the low efficiency of ATP utilization,

rapid glucose consumption may be indispensable. Therefore, suppression of undesirable side reactions such as ATPase reaction may enhance glutathione production.

Glutathione once produced was degraded after prolonged incubation (Fig. 6). So, to produce glutathione efficiently, the reaction time should be controlled, and if possible, the glutathione-degrading activities should be suppressed.

Lastly, NAD, the essential cofactor of the glycolytic pathway, was specifically released from the gel during the repeated use (Table 4). Therefore, to produce glutathione continuously, NAD supply is indispensable. The reason why ATP does not leak out from the gel is unknown.

Based on these considerations, continuous glutathione production was carried out using the immobilized *S. cerevisiae* cell column. However, the productivity of this system is somewhat low compared to the conventional fermentation method or chemical synthesis. By elevating the glutathione-producing activity of immobilized cells, this glutathione production system may be promising one.

SUMMARY

Whole cells of *Saccharomyces cerevisiae* were immobilized in polyacrylamide gel lattice. Consuming glucose, the immobilized cells produced glutathione from its constituent amino acids, and glutathione produced was excreted out of the gels. The conditions for immobilization of the yeast cells and for the glutathione production were studied. Based on these data, the properties and the feasibilities of the glycolytic pathway as an ATP regeneration system were discussed in reference to glutathione production.

Section 2 Glycolytic pathway as an ATP generation system and its application to the production of glutathione and NADP

INTRODUCTION

Construction of generating systems for ATP and/or coenzymes is very important for reaction efficiency and process economy in order to utilize enzymes in bioreactors for multi-step biosynthetic reactions. I have been studying the construction of an ATP-generating system for the production of glutathione. As shown in previous section, the glycolytic pathway of *S. cerevisiae* cells immobilized with polyacrylamide gel was found to be a promising and feasible ATP-generating system, judging from both the operational stability and ATP-generating activity.

To confirm the feasibility and to expand the field of application, the glycolytic pathway as an ATP-generation system was investigated using yeast cells immobilized in polyacrylamide gel. For efficient utilization of ATP generated by immobilized yeast cells, the reaction was coupled with transformation reactions for the production of glutathione and NADP. Thus, I investigated how the efficiency of ATP utilization is influenced by transformation reactions. For this investigation, two kinds of reactor systems, a co-immobilized cell system and a mixed-immobilized cell system, were employed for the production of glutathione and NADP. The co-immobilized cell system used *S. cerevisiae* cells immobilized together with other cells in the same polyacrylamide gel. On the other hand, in the mixed-immobilized cell system, *S. cerevisiae* and other cells were immobilized separately in polyacrylamide gel and the two kinds of immobilized

cells obtained were then mixed. In these reaction systems, the ATP-generating reaction and the transformation reaction were carried out by two different kinds of microbial cells. Namely, these systems were designed to make ATP transfer between the two kinds of microbial cells responsible for ATP generation and transformation.

This section deals with the properties, efficiencies and feasibility of ATP generation by immobilized *S. cerevisiae* cells and two kinds of reactor systems for the production of glutathione and NADP.

MATERIALS AND METHODS

Chemicals NAD was purchased from Oriental Yeast Co., Ltd., Tokyo, Japan. Adenosine and AMP were obtained from Kojin Co., Ltd., Tokyo, Japan. Other chemicals were all analytical grade reagents.

Cultivation and immobilization of microbial cells *Saccharomyces cerevisiae* (IFO 2044), *Escherichia coli* B (ATCC 23226) and *Brevibacterium ammoniagenes* (IAM 1645) were cultivated and immobilized individually with polyacrylamide gel as described in section 1 of this Chapter. For the co-immobilization in polyacrylamide gel, *S. cerevisiae* cells and other cells were homogeneously mixed in the ratio of 3:2 of wet weight, and then immobilized by the same procedures as described in section 1 of this Chapter. In all cases, 1.0 ml of gel contained 0.35 g (wet wt) intact cells.

Treatment of immobilized cells In order to improve the transport of substrates and/or products, immobilized cells were treated with toluene as follows. The immobilized cells (10 ml) were suspended in 100 ml of 5.0 mM potassium phosphate buffer (pH 7.0) containing 10 % toluene and 0.1 mM dithiothreitol, and incubated at 25 °C for 30 min with shaking. The immobilized cells were also treated with dithiothreitol or L-cysteine as follows. The immobilized cells (5.0 ml) were suspended in 50 ml of 5.0 mM potassium phosphate buffer (pH 7.0) containing 0.05 M dithiothreitol or 0.1 M L-cysteine, and incubated at 25 °C for 30 min with shaking.

Acetone treatment of intact cells The intact cells of *S. cerevisiae* (10 g wet wt.) were suspended in 10 ml of 0.85 % saline solution. To this cell suspension, 90 ml chilled acetone was added and stirred for 5 min. The cells were collected by filtration and dried on P₂O₅ in *vacuo*.

Reaction for ATP generation The ATP-generating reaction was carried out by incubating the immobilized cells (3 ml) in 10 ml of mixture containing 0.5 M glucose, 20 mM MgCl₂, 0.1 M potassium-phosphate buffer (pH 7.0), 1.0 mM AMP, 0.1 mM NAD and 10 mM adenosine at 30 °C with shaking. At prescribed times, 0.2 ml of reaction mixture was taken and glucose, FBP and ATP in the reaction mixture were determined. FBP and glucose were determined by the methods given by Roe et al.⁵⁰ and Hugget et al.⁵¹, respectively. ATP was determined by coupling with G-6-P dehydrogenase reaction.

Reaction for glutathione formation The glutathione-forming reaction was carried out by incubating the immobilized cells (0.5 ml) in 3.0 ml of reaction mixture containing 10 mM L-glutamate, 10 mM L-cysteine, 10 mM glycine, 10 mM MgCl₂, 50 mM potassium phosphate buffer (pH 7.0), 10 mM ATP, 10 mM creatine phosphate and 1.2 unit/ml creatine kinase at 30 °C with shaking. Periodically 0.01 ml aliquots of reaction mixture were taken and glutathione formed determined by the method of Tietze⁴⁹.

Reaction for NADP formation The NADP-producing reaction was performed by incubating the immobilized cells (0.5 ml) in 3.0 ml of reaction mixture containing 20 mM ATP, 20 mM NAD, 10 mM MgCl₂, and 50 mM potassium phosphate buffer (pH 7.0) at 30 °C with shaking. At prescribed times, 0.1 ml of reaction mixture was taken and the NADP formed was determined by the enzymatic method²⁵.

Reaction for glutathione production coupled with ATP generation The glutathione-producing reaction was performed by incubating the immobilized *E. coli* B cells (2.0 ml) or immobilized *S. cerevisiae* cells (3.0 ml) in 10 ml of mixture containing 0.5 M glucose, 10 mM MgCl₂, 0.1 M potassium phosphate buffer (pH 7.0), 1.0 mM AMP, 0.1 mM NAD, 10 mM adenosine, 20 mM L-glutamate, 20 mM L-cysteine, 20 mM glycine at 30 °C with shaking. For glutathione production by the co-immobilized cell system, 5.0 ml of co-immobilized cells of *S. cerevisiae* and *E. coli* B was incubated under the same conditions. In the case of glutathione production by mixed-immobilized cell system, both the immobilized *S. cerevisiae* cells (3 ml) and immobilized *E. coli* B cells (2 ml) were simultaneously incubated in the same reaction mixture. With time, 0.2 ml of reaction mixture was taken, and the concentration of ATP, FBP, glucose and glutathione were determined.

Reaction for NADP production coupled with ATP generation The NADP-producing reaction was carried out by incubating the immobilized *B. ammoniagenes* cells (2 ml) or immobilized *S. cerevisiae* cells (3 ml) in 10 ml of mixture containing 0.5 M glucose, 10 mM MgCl₂, 0.1 M potassium phosphate buffer (pH 7.0), 1.0 mM AMP, 10 mM NAD, 10 mM adenosine at 30 °C with shaking. For the production of NADP by the co-immobilized cell system, 5.0 ml of co-immobilized cells of *S. cerevisiae* and *B. ammoniagenes* cells were incubated. In the case of NADP production by the mixed-immobilized cell system, the immobilized *S. cerevisiae* cells and immobilized *B. ammoniagenes* cells (2 ml) were incubated simultaneously in the same reaction conditions. An aliquot (0.2 ml) of the reaction mixture was taken periodically and the concentration of ATP, FBP, glucose and NADP in the reaction mixture were determined.

Assay of ATP-degrading activity ATP-degrading activity was determined by incubating the immobilized cells (10 ml) in the 20 ml mixture containing 10 mM ATP, 10 mM $MgCl_2$, and 0.1 M potassium phosphate buffer (pH 7.0) at 30 °C with shaking for 1 h. Intact cells (0.35 g wet wt.) and acetone-dried cells (0.07 g dry wt.) were also incubated in the same reaction conditions. After 1 h of reaction, the residual ATP in reaction mixture was determined.

RESULTS

Properties of ATP-generating reaction

Figure 1 shows the time course of ATP generation by immobilized microbial cells with polyacrylamide gel. Immobilized *B. ammoniagenes* cells failed to generate ATP from adenosine (Fig.1C).

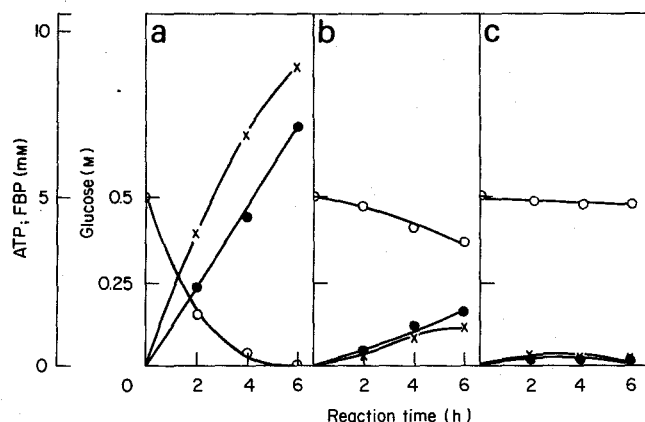


Figure 1 ATP generation by various immobilized microbial cells. The ATP-generating reaction was carried out under the standard assay conditions as described in Materials and methods except that 3.0 ml of immobilized cells was used. Immobilized cells used were: (a), immobilized *S. cerevisiae* cells; (b), immobilized *E. coli* cells; (c), immobilized *B. ammoniagenes* cells. ●, ATP; ×, FBP; ○, glucose

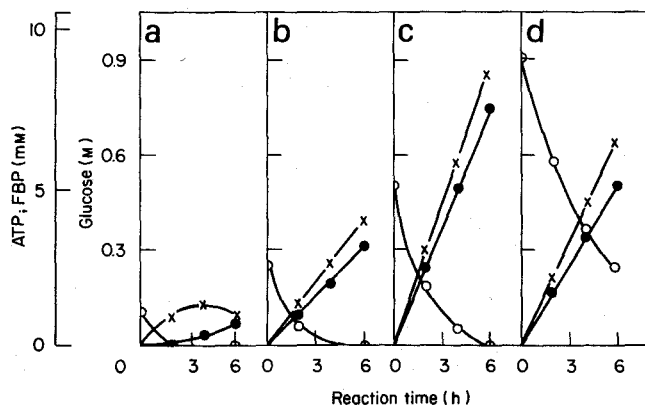


Figure 2 Effect of glucose concentration on ATP generation by immobilized *S. cerevisiae* cells. ATP-generating reaction was carried out under the standard assay conditions as described in Materials and methods except for the glucose concentration. Glucose concentrations: (a), 0.1 M; (b) 0.25 M; (c), 0.5 M; (d), 0.9 M. ●, ATP; ×, FBP; ○, glucose

On the other hand, the immobilized *S. cerevisiae* cells (Fig. 1A) and immobilized *E. coli* β cells (Fig. 1B) generated ATP from adenosine, consuming glucose and converting it to ethanol and carbon dioxide. The ATP-generating activity of immobilized *S. cerevisiae* cells was much higher than that of the immobilized *E. coli* β cells. Furthermore, the ATP-generating activity of immobilized *S. cerevisiae* cells was nearly equal to that of toluene-treated immobi-

lized *S. cerevisiae* cells, though the data are not shown here. To use the ATP-generating activity of immobilized *S. cerevisiae* cells for the production of useful compounds, detailed reaction conditions were studied further.

ATP generation from adenosine by immobilized *S. cerevisiae* cells

1) *Effect of glucose concentration* Figure 2 shows the effect of glucose concentration on ATP generation. ATP was formed efficiently at 0.5 M glucose concentration. FBP formation was also increased with the increasing glucose concentration up to 0.5 M.

2) *Effect of phosphate buffer concentration* As Figure 3 shows, the presence of higher phosphate buffer concentration favoured efficient ATP generation. Low phosphate buffer concentration reduced both ATP generation and FBP accumulation.

3) *Effect of $MgCl_2$ concentration* The optimal Mg^{2+} concentration for ATP generation by freshly prepared immobilized cells was around 5.0 mM.

4) *Effect of pH* In

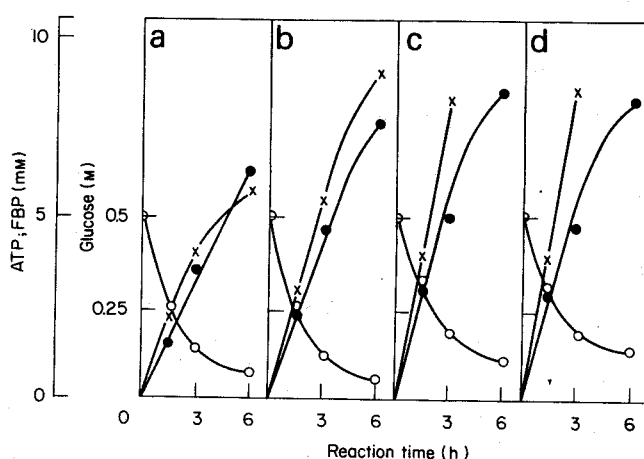


Figure 3 Effect of phosphate buffer concentration on ATP generation by immobilized *S. cerevisiae* cells. ATP-generating reaction was carried out under the standard assay conditions as described in Materials and methods except for the phosphate buffer concentration. Phosphate buffer concentrations: (a), 0.05 M; (b), 0.1 M; (c), 0.5 M; (d), 1.0 M. ●, ATP; ×, FBP; ○, glucose

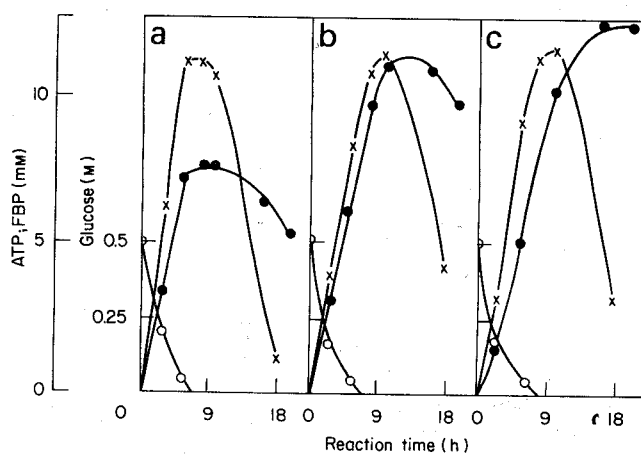


Figure 4 Effect of adenosine concentration on ATP generation by immobilized *S. cerevisiae* cells. ATP-generating reaction was carried out under the standard assay conditions as described in Materials and methods except for the adenosine concentrations. Adenosine concentrations: (a), 5.0 mM; (b), 10 mM; (c), 20 mM. ●, ATP; ×, FBP; ○, glucose

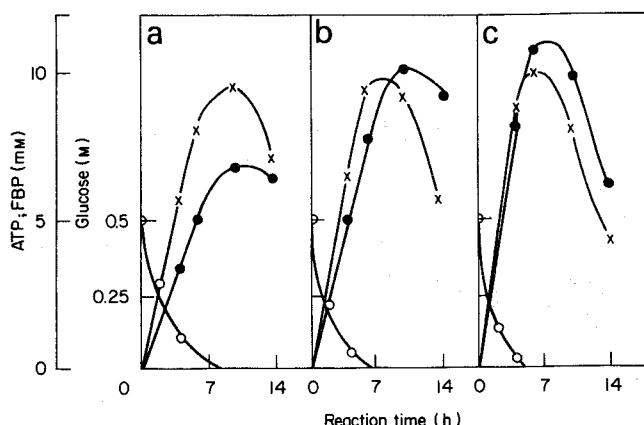


Figure 5 Effect of various amounts of immobilized *S. cerevisiae* cells on ATP generation. ATP-generating reaction was carried out under the standard assay conditions as described in Materials and methods except for the various amounts of immobilized cells that were used. Amount of immobilized cells: (a), 1.0 ml; (b), 2.0 ml; (c), 3.0 ml. ●, ATP; ×, FBP; ○, glucose

Table 1 ATP-degrading activity of various cell preparations of *S. cerevisiae*

Cell preparation	ATP-degrading activity ^a
Intact cells	0.43
Acetone-dried cells	47.5
Immobilized cells	16.1 (5.63) ^d
Reduced immobilized cells ^b	32.1
Reduced immobilized cells ^c	26.8

^a ATP degraded μmol per hour per g wet wt cells

^b Reduced with dithiothreitol

^c Reduced with L-cysteine

^d ATP-degrading activity expressed as $\mu\text{mol h}^{-1} \text{ml}^{-1} \text{gel}$. Reduction of immobilized cells were carried out as described in Materials and methods. ATP-degrading activity was determined as described in Materials and methods

the course of ATP generation, the decrease in pH of the reaction mixture was observed. The effect of initial pH was investigated, and the range of pH 7.0 to 7.5 was found to be most effective for ATP generation. Starting the reaction at this pH, the pH of reaction mixture was found to decrease to about 6.0 after complete consumption of 0.5 M glucose.

Effect of adenosine concentration Figure 4 shows the effect of adenosine concentration on

ATP generation. Adenosine at a concentration 5 to 10 mM was completely converted to ATP at the same velocity. At concentrations of adenosine higher than 10 mM, the rate of conversion of adenosine to ATP decreased.

Effect of immobilized cell concentration Figure 5 shows the time course for ATP generation by various quantities of immobilized cells. In all the cases, ATP concentration in a reaction mixture initially increased, reached a maximum, and then declined gradually. This change in ATP concentration was associated with the change in FBP accumulation in the reaction mixture. From the

results presented in Figure 5, the ATP-generating activity of immobilized cells was calculated to be 7.0 $\mu\text{mole/h/ml-gel}$, which corresponds to 18 $\mu\text{mole/h/g}$ wet cells.

ATP-degrading activity of immobilized cells

Table 1 shows the ATP-degrading activities of various cell preparations. By immobilizing the cells in polyacrylamide gel, the ATP-degrading activity was lowered to about a third that of acetone-dried cells. The treatment of immobilized cells with dithiothreitol or L-cysteine restored the deteriorated ATP-degrading activity of immobilized cells. The effect of dithiothreitol on the ATP-generating activity of immobilized cells was also investigated. As shown in Figure 6, by incubating the immobilized cells in the presence of 10 mM dithiothreitol, the ATP-gene-

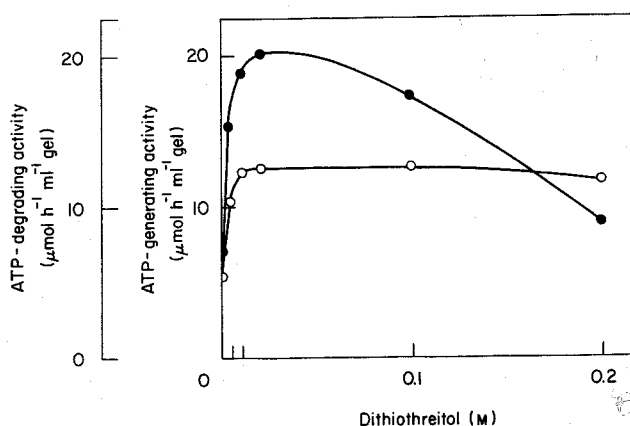


Figure 6 Effect of dithiothreitol on ATP-generating and ATP-degrading activities of immobilized *S. cerevisiae* cells. ATP-generating and ATP-degrading reactions were carried out for 1 h as described in Materials and methods in the presence of dithiothreitol at the concentrations indicated. ●, ATP-generating activity; ○, ATP-degrading activity

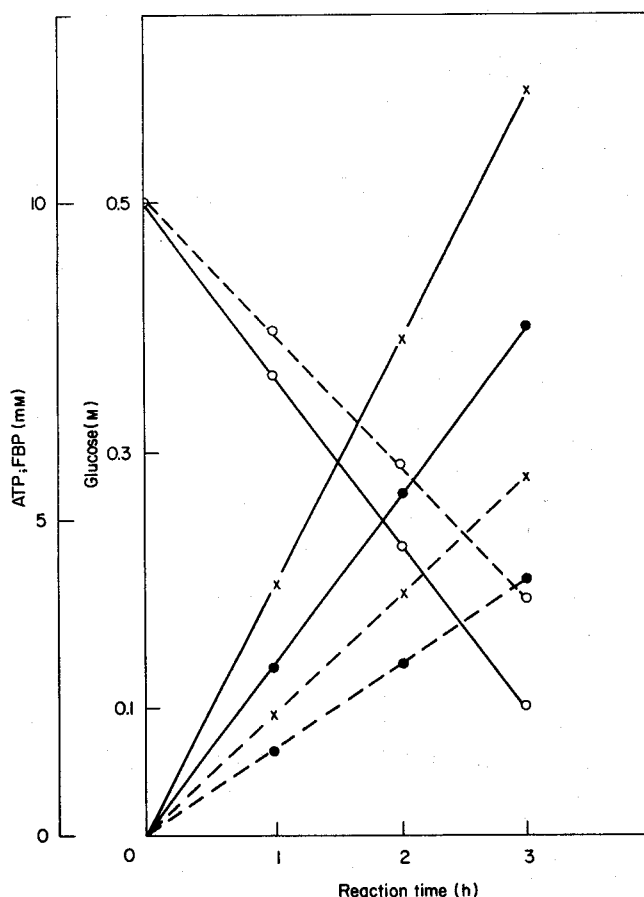


Figure 7 Effect of dithiothreitol on ATP-generating activity. ATP-generating reaction was carried out as described in Materials and methods in the presence (—) or absence (---) of 10 mM dithiothreitol. ●, ATP; ×, FBP; ○, glucose

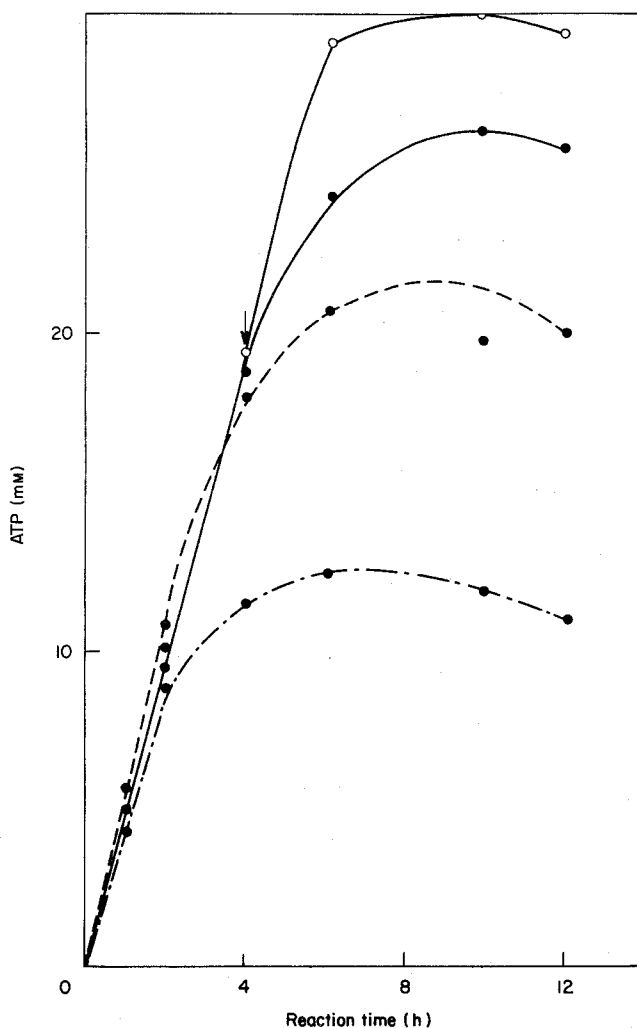


Figure 8 Effect of adenosine concentration on ATP generation by immobilized *S. cerevisiae* cells. ATP-generating reaction was carried out in the presence of 20 mM dithiothreitol. o, Parallel run of ATP generation from 30 mM adenosine, of which reaction mixture received 2.0 mmol solid glucose at 4 h as indicated by arrow. Other reaction conditions were the same as described in Materials and methods. Adenosine concentrations used were: - · - · -, 10 mM; - - - -, 20 mM; —, 30 mM

rating activity increased about two fold. In this case, not only the ATP-generating activity, but also the velocities of glucose consumption and FBP accumulation were found to be elevated simultaneously (Fig. 7). Furthermore, by carrying out the ATP-generating reaction in the presence of 20 mM dithiothreitol, larger amounts of adenosine were fully converted to ATP in a very short time (Fig. 8).

Continuous generation of ATP by immobilized cell column As indicated in Figures 4 and 5, the immobilized *S. cerevisiae* cells converted adenosine

to ATP in a batch reaction. To generate ATP continuously, the immobilized *S. cerevisiae* cells were packed in a column and substrate solution was passed through at various flow rates (Fig. 9). The maximum formation of ATP was attained at flow rates below space velocity (SV) = 0.1 h^{-1} , 30 °C. Figure 10 shows the continuous generation of ATP by the immobilized *S. cerevisiae* cell column. The conversion of adenosine to ATP was about 60 % and the half-life of this column was calculated to be 19 days.

Properties of transformation reactions

The properties of transformation reactions were investigated on the toluene-treated immobilized *E. coli* B cells and toluene-treated immobilized *B. ammoniagenes* cells responsible for the pro-

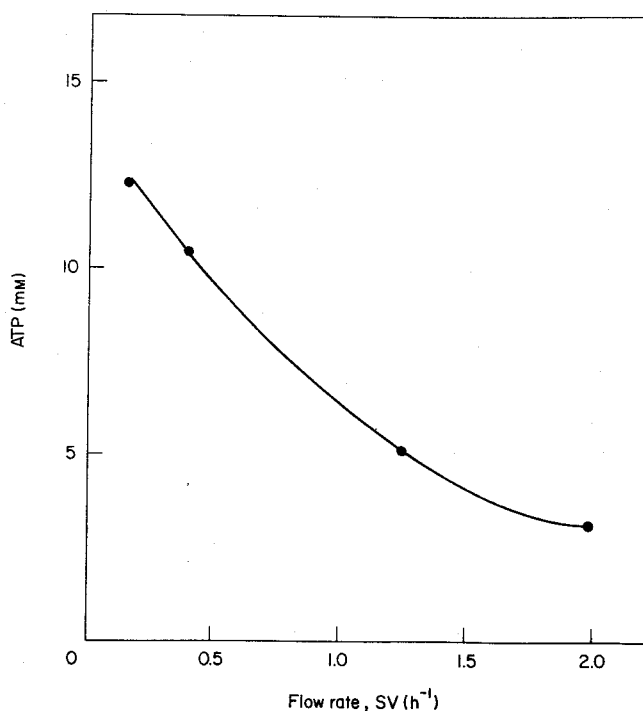


Figure 9 Effect of flow rates on ATP generation by immobilized *S. cerevisiae* cell column. The immobilized *S. cerevisiae* cells (5 ml) were packed in a column (1.0 X 10 cm) and substrate solution was passed upward through the column at various flow rates at 30°C. Substrate solution contained 15 mM adenosine, 0.1 mM NAD, 5.0 mM MgCl₂, 0.1 M potassium phosphate buffer, pH 7.0, 0.5 M glucose, 1.0 mM AMP and 1.0 mM dithiothreitol. ATP produced in effluent was determined

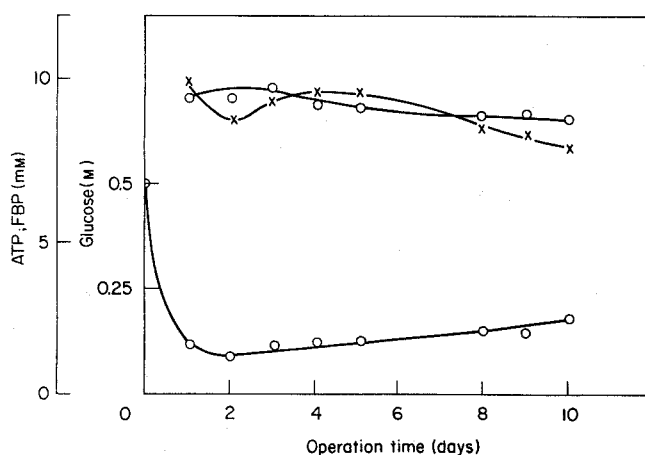


Figure 10 Continuous generation of ATP by immobilized *S. cerevisiae* cell column. Substrate solution as described in the legend to Figure 9 was continuously supplied to the column at SV = 0.3 h⁻¹, at 30°C. Other operational conditions were the same as in Figure 9. ●, ATP; X, FBP; ○, glucose

duction of glutathione and NADP, respectively.

1) Effect of pH

Figure 11 shows the effect of pH on the activities of transformations. The optimal pH for glutathione-synthesizing activity of immobilized *E. coli* B cells and for NAD kinase activity of immobilized *B. ammoniagenes* cells was 6.5 and 5.5, respectively.

2) Effect of Mg²⁺ concentration As shown in Figure 12, the optimal Mg²⁺ concentration for glutathione-synthesizing activity of immobilized *E. coli* B cells and NAD kinase activity of immobilized *B. ammoniagenes* cells were 30 and 5.0 mM, respectively.

3) Effect of phosphate buffer concentration

Figure 13 shows the effect of phosphate buffer con-

centration on glutathione synthesizing and NAD kinase activities of immobilized cells. The glutathione synthesizing activity of immobilized *E. coli* B cells was severely inhibited by phosphate buffer, though the NAD kinase activity of immobilized *B. ammoniagenes* cells was not inhibited.

4) Kinetic studies on transformation reactions

The kinetic studies on glutathione-synthesizing enzymes in immobilized *E. coli* B cells and NAD kinase in immobilized *B. ammoniagenes* cells were performed using ATP as described in Materials and Methods. For the assay of glutathione-synthesizing enzymes, the glutathione-forming reaction was carried out in the presence of ATP regeneration system consisting of creatine kinase. Under these conditions, immobilized *E. coli* B cells formed glutathione ef-

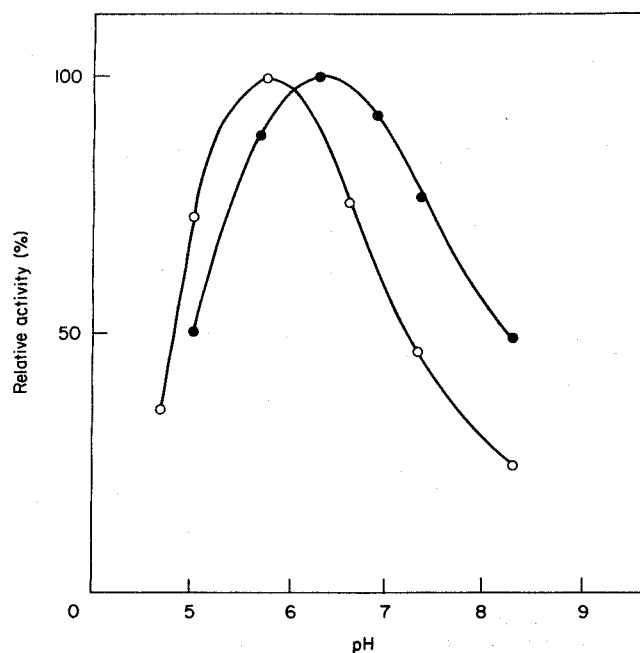


Figure 11 Effect of pH on glutathione formation by immobilized *E. coli* cells (●) and on NADP formation by immobilized *B. ammoniagenes* cells (○). Glutathione- and NADP-forming reactions were carried out as described in Materials and methods except for the pH of reaction mixture. The pH of reaction mixture was adjusted with 0.1 M sodium acetate buffer (pH 4.7–6.0) or 0.1 M potassium phosphate buffer (pH 6.0–8.4). The enzyme activities at pH 5.7 and 6.5 were taken as 100% for activities of NAD kinase and glutathione-forming enzymes, respectively

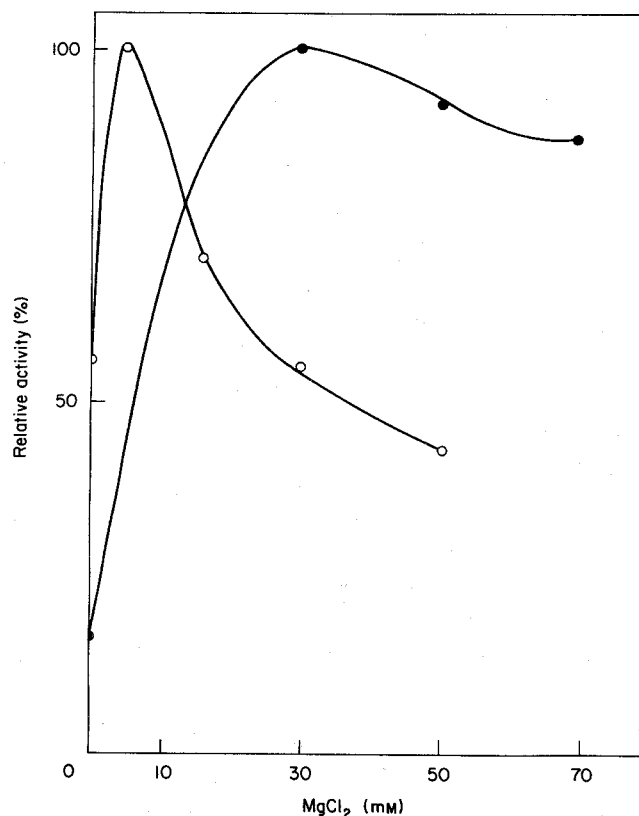


Figure 12 Effect of Mg^{2+} concentration on glutathione formation by immobilized *E. coli* cells (●) and on NADP formation by immobilized *B. ammoniagenes* cells (○). Glutathione- and NADP-forming reactions were carried out as described in Materials and methods except for the Mg^{2+} concentration. The enzyme activities at 5 and 30 mM Mg^{2+} were taken as 100% for activities of NAD kinase and glutathione forming enzymes, respectively

Table 2 Properties of ATP-generating reaction and transformation reaction

Reaction system	Enzyme	Microbial cells	Optimal conditions for				Kinetic parameter			
			Glucose	Mg ²⁺	Phosphate buffer	pH	K _m for			Specific activity (μmol h ⁻¹ ml ⁻¹ · gel)
			(M)	(mM)	(M)		ATP (mM)	NAD (mM)	L-Cysteine (mM)	
ATP generation	Glycolytic pathway	<i>S. cerevisiae</i>	0.5	5.0	<0.5	7–7.5	–	–	–	7.0
Transformation	γ-Glutamylcysteine synthetase and glutathione synthetase	<i>E. coli</i>	–	30	>0.05	6.5	5.0	–	3.3	2.1
		<i>S. cerevisiae</i> ^a	–	20	>0.05	6.5	N.d.	–	14.0	1.3
Transformation	NAD kinase	<i>B. ammoniagenes</i>	–	5.0	(independent)	5.5	3.3	4.0	–	0.65
		<i>S. cerevisiae</i>	–	N.d.	N.d.	N.d.	14.1	4.3	–	0.17

^a Data on *S. cerevisiae* cells are taken from ref. 5

^b N.d. Not determined

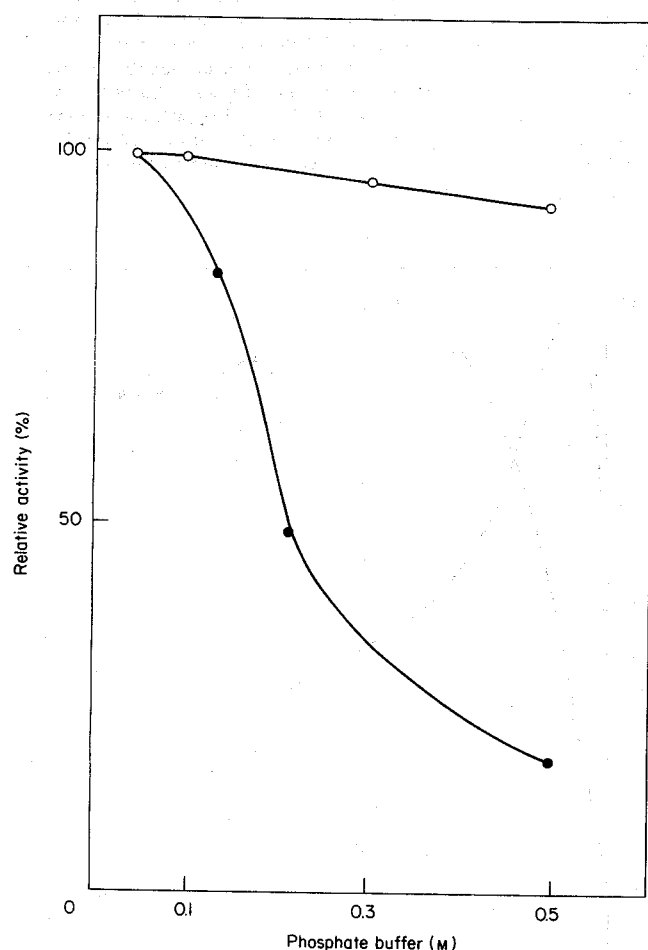


Figure 13 Effect of phosphate buffer concentration on glutathione formation by immobilized *E. coli* cells (●) and on NADP formation by immobilized *B. ammoniagenes* cells (○). Glutathione- and NADP-forming reactions were carried out as described in Materials and methods except for the phosphate buffer concentration. The activities in 0.05 M phosphate buffer were taken as 100% for the activities of glutathione forming enzymes and NAD kinase

ficiently and the glutathione forming activity was determined to be 2.1 μmole/h/ml-gel (Table 2). The K_m values for L-cysteine and ATP were also determined to be 3.3 and 5.0 μmole/h/ml-gel, respectively. The K_m values for amino acids other than L-cysteine were not determined because the overall reaction rate of glutathione formation was strongly dependent on the L-cysteine concentration (data not shown). On the other hand, the NAD kinase reaction proceeded well without the ATP regeneration system, as mentioned above. The NADP forming activities of immobi-

lized *B. ammoniagenes* cells and immobilized *S. cerevisiae* cells

were determined to be 0.65 and 0.17 $\mu\text{mole/h/ml-gel}$, respectively. The K_m values for ATP and NAD are summarized in Table 2.

Production of glutathione and NADP by coupled reaction systems of ATP generation and transformation

1) *Reactor systems for the production of glutathione and NADP* For the production of glutathione and NADP, ATP-generating and transformation reactions were combined into two kinds of reactor systems (Fig. 14). In these reactor systems, ATP generation from adenosine, AMP and ADP is carried out by *S. cerevisiae* cells immobilized in polyacrylamide gel. Transformation reactions are carried out by microbial cells other than *S. cerevisiae* cells, and substrate (S) is transformed to product (P) using ATP generated by immobilized *S. cerevisiae* cells. To compare the efficiencies of ATP utilization, two kinds of reactor systems were employed. One is mixed-immobilized cell system (Fig. 14a), this system represents the mixed use of *S. cerevisiae* cells and other cells separately immobilized in polyacrylamide gel. The other is a co-immobilized cell system (Fig. 14b). This system represents the mixed use of *S. cerevisiae* cells immobilized together with other cells in the same polyacrylamide gel. Unless otherwise noted, in the following experiments, the immobilized cells represent the toluene-treated immobilized cells.

2) *Production of glutathione and NADP* Figures 15 and 16 show the time course of glutathione and NADP production by various immobilized cells. Consuming glucose, the immobilized *S. cerevisiae* cells (Fig. 15a) and immobilized *E. coli B* cells (Fig. 15b) produced glutathione, although the activity of the latter was low. However, markedly higher efficiencies were not observed by coupling *S. cerevisiae* cells with *E. coli B* cells

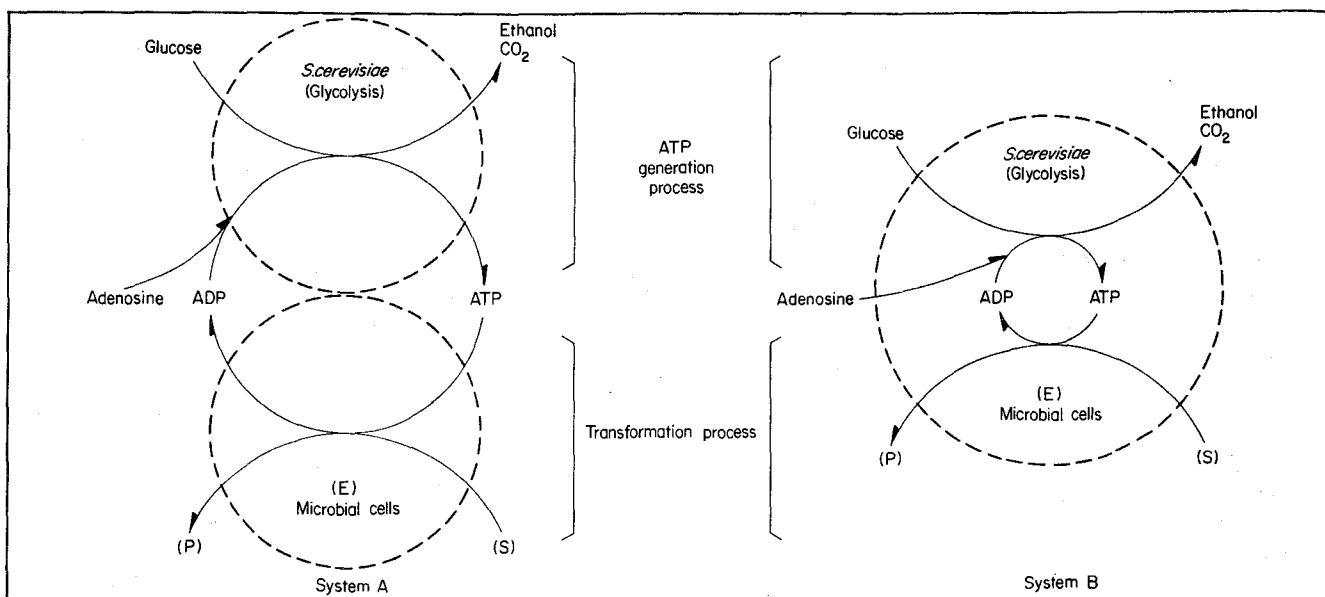


Figure 14 Schematic representation of the reactor systems. System A and System B represent the mixed immobilized cell system and the co-immobilized cell system, respectively. (S), (P), and (E) represent the substrate, product, and enzymes respectively, in transformation reactions, and they are summarized in the Table. Broken line shows the polyacrylamide gel lattice. Other details in reactor systems are described in the Results

Microbial cells	Enzymes	Substrate	Product
<i>E. coli</i>	γ -Glutamylcysteine synthetase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3)	L-Glutamate L-Cysteine Glycine	Glutathione
<i>B. ammoniagenes</i>	NAD kinase (EC 2.7.1.23)	NAD	NADP

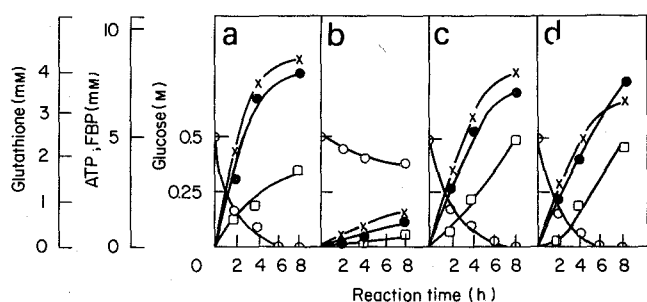


Figure 15 Time course of glutathione production by various immobilized cell preparations. Reaction was carried out using (a) immobilized *S. cerevisiae* cells; (b), immobilized *E. coli* cells; (c), co-immobilized *S. cerevisiae* cells with *E. coli* cells (co-immobilized cell system); (d), immobilized *S. cerevisiae* cells and immobilized *E. coli* cells (mixed immobilized cell system). ●, ATP; ×, FBP; ○, glucose; □, glutathione

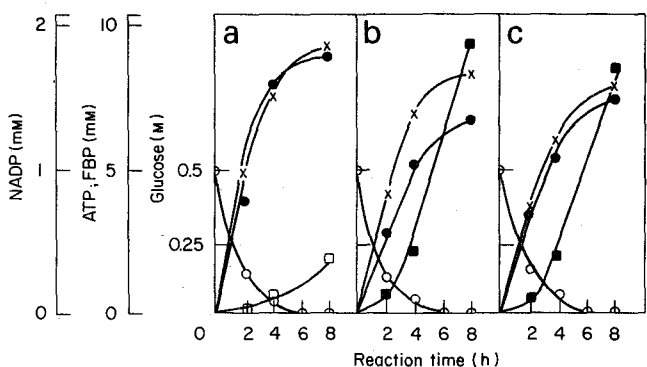


Figure 16 Time course of NADP production by various immobilized cell preparations. Reactions used (a), immobilized *S. cerevisiae* cells; (b), co-immobilized *S. cerevisiae* cells with *B. ammoniagenes* cells (co-immobilized cell system); (c), immobilized *S. cerevisiae* cells and immobilized *B. ammoniagenes* cells (mixed immobilized cell system). ●, ATP; ×, FBP; ○, glucose; ■, NADP

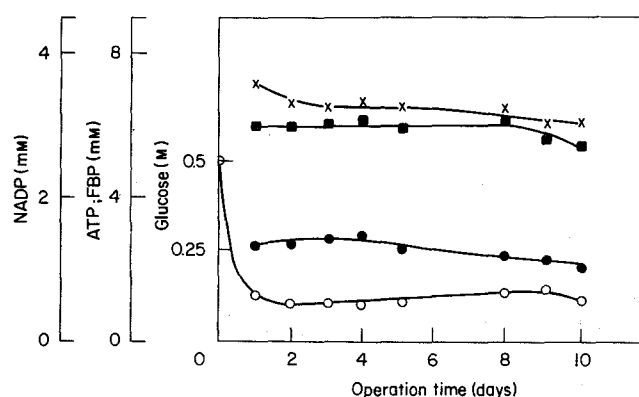


Figure 17 Continuous production of NADP by co-immobilized *S. cerevisiae* with *B. ammoniagenes* cell column. Co-immobilized *S. cerevisiae* cells with *B. ammoniagenes* cells (5 ml) were packed in a column (1.0 × 10 cm) and substrate solution was continuously passed upwards through the column at SV = 0.3 h⁻¹, at 30°C. Substrate solution contained 0.5 M glucose, 5 mM MgCl₂, 0.1 M potassium phosphate buffer, pH 7.0, 10 mM NAD, 1.0 mM AMP, 1.0 mM dithiothreitol and 15 mM adenosine. ●, ATP; ×, FBP; ○, glucose; ■, NADP

in both reactor systems (Fig. 15c and 15d). On the other hand, in the case of NADP production, a markedly higher efficiency was observed by coup-

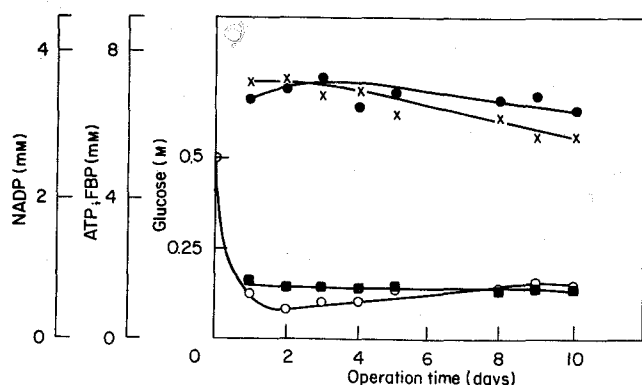


Figure 18 Continuous production of NADP by immobilized *B. ammoniagenes* cell column combined with immobilized *S. cerevisiae* cell column. The exit of the column packed with immobilized *S. cerevisiae* cells (5 ml) was directly combined with the entrance (i.e. bottom) of the column packed with immobilized *B. ammoniagenes* cells (5 ml). The substrate solution as described in legend to Figure 17 was continuously passed upwards through the immobilized *S. cerevisiae* cell column at $SV = 0.3 \text{ h}^{-1}$, at 30°C , and the products in effluent from the immobilized *B. ammoniagenes* cell column were determined. ●, ATP; ×, FBP; ○, glucose; ■, NADP

ling *S. cerevisiae* cells with *B. ammoniagenes* cells, though the differences in efficiencies of the two reactor systems were slight (Figs. 16b and 16c).

Production of NADP was investigated using the column of the co-immobilized cell system, and NADP was found to be produced continuously by this system, although the amount produced was low (Fig. 17). In this case, how-

ever, a large amount of ATP was also found in the effluent which had not been utilized by the NAD kinase reaction. To utilize as much as possible, the following NADP production method consisting of two columns was employed. The first column was packed with immobilized *S. cerevisiae* cells, and the second column contained immobilized *B. ammoniagenes* cells. The top of the first column was joined with the bottom of the second column so that the effluent from the first column was introduced directly into the second column at the same flow rate. By employing this method, NADP was efficiently produced in amounts exceeding those produced by the co-immobilized cell column (Fig. 18). On the other hand, investigations of the continuous production of glutathione presently offer no advantages, since the coupling effect of *S. cerevisiae* cells with *E. coli* β cells was not so great.

Table 3 Comparison of productivities of glutathione and NADP by single immobilized cell system and co-immobilized or mixed-immobilized cell system

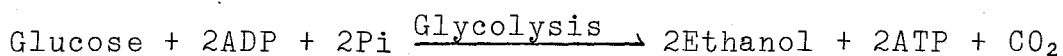
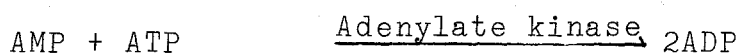
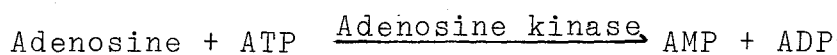
Product	Immobilized microbial cells used for		Producing activity ($\mu\text{mol h}^{-1} \text{ml}^{-1} \text{gel}$) ^a		
	ATP-generation	Transformation	Single immobilized cell system	Co-immobilized cell system	Mixed-immobilized cell system
Glutathione	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>	0.582	—	—
	<i>E. coli</i>	<i>E. coli</i>	0.082	—	—
	<i>S. cerevisiae</i>	<i>E. coli</i>	—	0.783	0.812
NADP	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>	0.050	—	—
	<i>B. ammoniagenes</i>	<i>B. ammoniagenes</i>	0	—	—
	<i>S. cerevisiae</i>	<i>B. ammoniagenes</i>	—	0.627	0.605

^a Activities were calculated from the data in Figures 15 and 16

DISCUSSION

I have been utilizing the immobilized *S. cerevisiae* cells for the production of glutathione. By regenerating the endogenous ATP via the glycolytic pathway, the immobilized *S. cerevisiae* cells produced glutathione from its constituent amino acids. However, the amount of ATP utilized for glutathione synthesis was only 0.5 % of the ATP regenerated by the glycolytic pathway consuming 0.5 M glucose. This poor efficiency of ATP utilization could be caused by the following reasons: (a) degradation of ATP by ATP-hydrolyzing enzymes such as ATPase or phosphatases: (b) low affinity of immobilized glutathione synthetic enzymes for ATP: (c) compartmentalization of the sites between the glycolytic pathway and the glutathione synthesizing reaction. Such compartmentalization may be a barrier to efficient ATP transfer. For the solution to these problems, I employed two kinds of reactor systems which improve the efficiency of ATP utilization. These were the mixed-immobilized cell system and co-immobilized cell system. In these systems, ATP-generating and transformation reactions were carried out by two different kinds of microbial

cells. That is, most ATP was generated by immobilized *S. cerevisiae* cells, and the ATP thus generated was transferred to other microbial cells responsible for the transformation reaction. To enable ATP transfer between two kinds of microbial cells, and to retain ATP in the reactor, adenosine was introduced into the reaction system, since *S. cerevisiae* cells can convert adenosine to ATP using endogenous ATP or exogenously added ATP at the catalytic concentration. The reaction scheme for ATP generation from adenosine was as follows:



Thus, I studied glutathione production with the two reactor systems. To compare the efficiencies and feasibilities of the two kinds of reactor systems, NADP production was also investigated in addition to glutathione production.

In the reactor systems employed, for glutathione and NADP production, the ATP-generating activity of immobilized *S. cerevisiae* cells seemed to be the important factor making these two kinds of reactor systems viable and feasible. As shown in Figures 4 and 5, adenosine was effectively converted to ATP by immobilized *S. cerevisiae* cells and continuous generation of ATP was also successfully carried out by using the immobilized *S. cerevisiae* cell column (Fig. 10). In batchwise reactions (Figures 4 and 5), ATP generation was associated with FBP accumulation and ATP, once generated, was gradually degraded with the decrease in FBP concentration in the reaction mixture. This phenomenon indicates

that FBP accumulation is essential for ATP generation and the ATP generated by FBP degradation is utilized preferentially for the conversion of adenosine, AMP and ADP to ATP. Thus, the glycolytic pathway of immobilized *S. cerevisiae* cells worked well as an ATP generation system.

In addition, the method of ATP generation using *S. cerevisiae* cells immobilized with polyacrylamide gel seems very useful, since by immobilizing the intact cells in a polyacrylamide gel lattice, the preparation having high ATP-generating activity was readily obtained. The acetone treatment or air-drying procedures required for the improvement of membrane permeability of adenosine nucleotide^{53,54,55)} was unnecessary in the case of immobilization with polyacrylamide gel. Furthermore, the ATP-degrading activity was lost by immobilization. The decrease in ATP-degrading activity seems to be due to the inactivation of sulfhydryl enzymes such as ATPase, since the treatment of immobilized cells with dithiothreitol or L-cysteine resulted in a partial increase in ATP-degrading activity (Table 1). Associating with the increase in ATP-degrading activity, however, the ATP-generating activity of immobilized cells also increased in the presence of dithiothreitol (Fig. 6). This increase in ATP-generating activity seems to be due to the simultaneous increase in glucose consumption and FBP accumulation (Fig. 7). In the continuous generation of ATP by acetone-treated yeast cells immobilized with photocrosslinkable resin, Asada et al.⁵³⁾ suggested that the effect of dithiothreitol was due to the protection of enzymes involved in the ATP-generating reaction. Besides the protective effect on enzyme inactivation, the enhancement of glycolytic activity must be taken into account. The effect of dithiothreitol suggests that the maintenance of the redox poten-

tial at a lower level is necessary for the efficient utilization of the glycolytic pathway as a tool for ATP generation. In fact, in the presence of dithiothreitol, a higher concentration of adenosine was efficiently and completely converted to ATP in a very short time (Fig. 8).

Judging from the kinetic data of transformation reactions shown in Table 2, the amount of ATP generated by batchwise or continuous procedures was high enough to saturate the transformation reactions. So, efficient glutathione and NADP production was expected by coupling the ATP-generating reaction with the transformation reactions. However, in the case of glutathione production, the coupling effect of *S. cerevisiae* cells with *E. coli* B cells resulted in a slight increase in the amount of glutathione by the reactor systems employed, the co-immobilized cell system and mixed-immobilized cell system (Figs. 15c and 15d). This seems to be due to the inhibition of γ -glutamylcysteine synthetase⁵⁶⁾ with ADP formed from adenosine introduced into the reaction system. On the other hand, NADP was efficiently produced by coupling *S. cerevisiae* cells with *B. ammoniagenes* cells, though there were no differences in the efficiency of the two reactor systems (Figs. 16b and 16c). Thus, the NADP production systems were easily applied to the continuous process using the co-immobilized cell column (Fig. 17). Further, by employing the two-column method, NADP production increased considerably (Fig. 18).

As summarized in Table 3, the productivity of NADP by the mixed- or co-immobilized cell system was much higher than that of the single immobilized cell system. But a marked increase in productivity by the mixed- or co-immobilized cell system was not observed in the production of glutathione. As to the factors determining these differences in production of glutathione and NADP,

the properties of the ATP-generating and transformation reactions must be considered. As Table 2 shows, except for optimal pH, the properties of the NAD kinase reaction roughly correspond to those of the ATP-generating reaction. So, good efficiency of NADP production was attained (Figs. 16b and 16c). The properties of glutathione synthesizing reaction were different from those of the ATP-generating reaction, as typically seen in the effect of phosphate buffer concentration (Fig. 13). Besides these differences in properties, there are other problems with using the enzyme reaction for glutathione synthesis. These are: (1) feedback inhibition of γ -glutamylcysteine synthetase by reduced glutathione; (2) inhibition of γ -glutamylcysteine synthetase by ADP; (3) glutathione-degrading activity of immobilized cells; (4) a glutathione transport barrier across the cell membrane. Some of them have already been overcome, but the inhibitory effect of phosphate on the glutathione synthetic reaction seems to be the barrier to further development of the glutathione production system utilizing the glycolytic pathway as a tool for ATP generation,

Nevertheless, the glycolytic pathway has been reported to be effective as an ATP generation system for the production of various useful compounds such as glutathione, NADP and S-adenosyl-L-methionine⁵⁷⁾. The glycolytic pathway has also been utilized for the production of ATP⁵³⁾ and cytidine diphosphate choline^{54,55)}.

SUMMARY

Efficient ATP generation is required to produce glutathione and NADP. Hence, the generation of ATP was investigated using the glycolytic pathway of yeast. *Saccharomyces cerevisiae* cells immobilized using polyacrylamide gel generated ATP from adenosine, consuming glucose and converting it to ethanol and carbon dioxide.

Under optimal conditions, the ATP-generating activity of immobilized yeast cells was 7.0 $\mu\text{mole/h/ml-gel}$. A column packed with these immobilized yeast cells was used for continuous ATP generation. The half-life of this column was 19 days at a space velocity (SV) of 0.3 h^{-1} at 30°C . The properties of glutathione- and NADP-producing reactions coupled with the ATP-generating reaction were investigated. *Escherichia coli* B cells with glutathione synthesizing activity and *Brevibacterium ammoniagenes* cells with NAD kinase activity were immobilized in a polyacrylamide gel lattice. Under the optimal conditions, the immobilized *E. coli* B cells and immobilized *B. ammoniagenes* cells produced glutathione and NADP at the rates of 2.1 and 0.65 $\mu\text{mole/h/ml-gel}$, respectively, adding ATP to the reaction mixture. In order to produce glutathione and NADP economically and efficiently, the glutathione- and NADP-producing reactions were finally coupled with the ATP-generating reaction catalysed by immobilized *S. cerevisiae* cells. To compare the productivities of glutathione and NADP and to compare the efficiency of ATP utilization for the production of these two compounds, the two reactor systems, co-immobilized cell system and mixed-immobilized cell system, were designed. As a result, these two compounds were also found to be produced by these two kinds of reactor systems. Using the data obtained, the feasibility and properties of ATP generation by immobilized yeast cells are discussed in terms of the production of glutathione and NADP.

CHAPTER III

Application of acetate kinase reaction as ATP regeneration system for production of glutathione

Section 1. Application of immobilized ATP in production of glutathione by multienzyme system

INTRODUCTION

At present, the use of enzymes in reactor systems is mainly limited to simple processes such as hydrolytic conversions. But many enzymatic processes require the participation of coenzymes and/or the availability of an energy supply (ATP). Therefore, it is necessary to design systems for regeneration of coenzymes and/or ATP in order to use such enzymes economically in reactor systems.

For this purpose, many coenzymes and ATP have been immobilized by attachment to water-soluble polymers to retain them in reactors. The usefulness of immobilized coenzymes has been well demonstrated, especially in dehydrogenase systems linked with NAD or NADP⁵⁸⁻⁶⁸). While immobilized ATP has been used for the affinity chromatographic separation of specific proteins, its use as cosubstrate in enzyme reactor systems has not been extensively investigated⁶⁹⁻⁷²).

As a step in the development of reactor systems involving ATP-requiring enzymes, I have investigated the use of ATP bound to a water-soluble polymer, dextran, for the production of glutathione. In the present study, the possibility of recycling of ATP was examined using dextran-bound ATP co-immobilized with whole *Escherichia coli* B cells, which contain all of the enzymes responsible for glutathione synthesis and ATP regeneration.

MATERIALS AND METHODS

Materials ATP was obtained from Kojin company, Ltd., Tokyo, Japan. Acetyl phosphate was purchased from Sigma Chemical Company, St. Louis, Missouri. Dextran (M.W. 4,0000) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Preparation of ATP analogues N^6 -Carboxymethyl ATP (N^6 -R'-ATP), N^6 -((6-aminoethyl)carboxymethyl)-ATP (N^6 -R"-ATP), and dextran-bound N^6 -R"-ATP were synthesized as described by Mosbach et al.⁶⁹. Samples of dextran-bound N^6 -R"-ATP differing in the binding number to dextran of N^6 -R"-ATP were synthesized using different amounts of CNBr. Thus samples of binding number 41.0, 64.3, and 91.2 $\mu\text{mol/g}$ dextran were obtained by addition of 0.8 mmol N^6 -R"-ATP to 1.0 g dextran activated with CNBr in the amounts of 0.2, 0.4 and 0.8 mmol, respectively. Binding number determinations were based on the molar absorption coefficient of N^6 -R"-ATP (17300 $\text{M}^{-1} \text{cm}^{-1}$, $\lambda_{\text{max}}=267 \text{ nm}$ in 0.01 M HCl) and the concentration of dextran. The concentration of dextran was determined by the method of Dubois et al.⁷³.

Preparation of cell extracts of E. coli B Cell extracts of *E. coli B* (ATCC 23226) were prepared as follows. *E. coli B* cells grown on the nutrient medium (medium A shown in section 1-1 of Chapter I) were harvested and washed once with sodium chloride solution (0.85 g/100 ml). Cells were suspended in 5.0 mM Tris-HCl buffer (pH 7.0) containing 0.5 mM L-cysteine and sonicated for 5 min at 9 kHz at 0°C. The resultant suspension was centrifuged at 25,000 $\times g$ for 20 min. Cell extracts thus obtained were used as the source of acetate kinase and glutathione-forming enzyme system. Protein was determined by the method of Lowry et al.²⁶.

Immobilization of dextran-bound N^6 -R"-ATP and whole E. coli B cells

Dextran-bound N^6 -R"-ATP (binding number: 64.3 $\mu\text{mol/g}$ dextran) was co-immobilized with whole *E. coli B* cells in polyacrylamide gel. Polyacrylamide gel was prepared as described by Chibata et al.³⁴. Gel-A (1.0 ml) contained dextran-bound N^6 -R"-ATP (150 mg) and intact *E. coli B* cells (0.2 g wet wt). Gel-B (1.0 ml) contained intact *E. coli B* cells (0.2 g wet wt) only. Following preparation as above, Gel-A and Gel-B were shaken in 5.0 mM Tris-HCl buffer (pH 7.5) containing 10 % (v/v) toluene and 0.5 mM L-cysteine for 30 min at room temperature. Unless otherwise noted, Gel-A and Gel-B represent toluene-treated Gel-A and toluene-treated Gel-B.

Determination of cosubstrate activities of ATP analogues for acetate kinase and glutathione-forming enzyme system

The cosubstrate activities of N^6 -R'-ATP, N^6 -R"-ATP, and dextran-bound N^6 -R"-ATP for acetate kinase and glutathione-forming enzyme system were determined as follows. The cosubstrate activities of ATP analogues for acetate kinase were determined using the mixture (2.5 ml) containing 0.6 M acetate, 5.0 mM MgCl_2 , 4.0 M NH_2OH , 10 mM ATP analogue, 0.1 M Tris-HCl buffer (pH 7.5), and cell extract (0.1-0.2 mg/ml protein) or 1.0 ml gel. Reaction was carried out at 37 °C for 30 s and hydroxamate formed was determined by the method of Lipman et al.⁷⁴. The co-

substrate activities of ATP analogues for glutathione-forming enzyme system were determined using the mixture (2.5 ml) containing 20 mM L-glutamate, 20 mM L-cysteine, 20 mM glycine, 10 mM MgCl_2 , 20 mM ATP analogue, 20 mM acetyl phosphate, 0.1 M Tris-HCl buffer (pH 7.0), and cell extract (2.0-4.0 mg/ml protein) or 1.0 ml gel. Reaction was carried out at 37 °C for 60 min and glutathione formed was determined by the method of Tietze⁴⁹. Acetate kinase and glutathione-forming activities were assayed using ATP instead of ATP analogue in reaction mixtures of the same composition. Cell or immobilized cell homogenates were prepared by the method described in section 1-3 of Chapter I, and they were also used as enzyme sources.

RESULTS

Cosubstrate activities of ATP analogues

The cosubstrate activities of free ATP analogues and dextran-bound $\text{N}^6\text{-R''-ATP}$ for acetate kinase and the glutathione-forming enzyme system were compared with that of ATP (Table 1). Substitution at position N^6 of the adenine ring reduced the activity to 40-50 % of that of ATP in the case of acetate kinase and to 10-50 % in the case of glutathione-forming enzyme system. The attachment of $\text{N}^6\text{-R''-ATP}$ to dextran further decreased its activity to about 15-35 % in the case of acetate kinase and to 10-30 % in the case of glutathione-forming enzyme system. The cosubstrate activity of dextran-bound $\text{N}^6\text{-R''-ATP}$ for acetate kinase and glutathione-forming enzyme system was dependent on the binding number of $\text{N}^6\text{-R''-ATP}$ to dextran, the cosubstrate activity being higher for the derivatives with low binding number. Dextran itself at the concentration used showed no appreciable effect on enzyme activities (Table 1).

Reactor system for glutathione production

Figure 1 shows the reactions involved in glutathione production. The overall process involves two reaction systems. The acetate kinase system converts dextran-bound $\text{N}^6\text{-R'-ATP}$ into

TABLE I
Cosubstrate Activity of ATP Analogs

Enzyme	Activity ^a					
	ATP	N ⁶ -R'-ATP	N ⁶ -R''-ATP	Dextran-bound N ⁶ -R''-ATP ^b		
				I	II	III
Acetate kinase	100 (107) ^c	43.1	56.2	35.6	33.4	16.1
Glutathione-forming enzyme system	100 (104) ^c	12.3	41.9	29.6	25.7	11.3

^a All values are quoted relative to ATP (100).

^b Binding numbers were 41.0 (I), 64.3 (II), and 91.2 (III) $\mu\text{mol/g}$ dextran.

^c Activity in the presence of 100 mg/ml dextran. Other reaction conditions are described under Materials and Methods.

dextran-bound N⁶-R''-ATP using acetyl phosphate as phosphate donor. The glutathione-forming enzyme system synthesizes glutathione from its three constituent amino acids consuming dextran-bound N⁶-R''-ATP in the process. *E. coli* B cells which contain the enzymes required for both reaction systems were immobilized in polyacrylamide gel together with dextran-bound N⁶-R''-ATP.

Activities of gel-entrapped enzymes

The activities of gel-entrapped acetate kinase and glutathione forming enzyme system were determined using Gel-B and compared with the activities of various cell preparations (Table II). The measured activities of acetate kinase and glutathione-forming

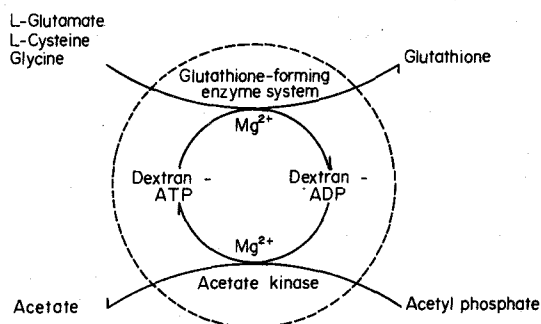


FIG. 1. Schematic representation of reactor for glutathione production. Dextran-ATP and dextran-ADP represent dextran-bound N⁶-R''-ATP and -ADP. Dotted line represents the polyacrylamide gel lattice. Other details are given under Results.

TABLE 2
Enzyme Activities of Various Cell Preparations

Enzyme	Activity ^a							
	Intact cells	Toluene-treated cells	Homogenate of cells (1)	Immobilized cells	Toluene-treated immobilized cells (2)	Homogenate of toluene-treated immobilized cells (3)	Activity yield ^b (%)	En-trapped activity ^c (%)
Acetate kinase	22.3	2140	2160	121	1125	1548	71.7	72.7
Glutathione-forming enzyme system	0.11	3.28	5.62	0.21	3.31	4.68	83.2	70.7

^a μmol of acetyl phosphate or glutathione produced/g cells/h.

^b $100 \times [(3)/(1)]$.

^c $100 \times [(2)/(3)]$. Other reaction conditions are described under Materials and Methods.

enzyme system in the gel were markedly increased by toluene treatment. The intrinsic activities of these enzymes in *E. coli* B cells were determined in cell homogenates and were found to be 2160 $\mu\text{mol/g cells/h}$ for acetate kinase and 5.62 $\mu\text{mol/g cells/h}$ for glutathione-forming enzyme system. The activities in the homogenate of toluene-treated immobilized cells were 1548 $\mu\text{mol/g cells/h}$ for acetate kinase and 4.68 $\mu\text{mol/g cells/h}$ for glutathione-forming enzyme system. From these values, the activity yield ((activity of homogenized toluene-treated immobilized cells/ activity of cell homo-

TABLE 3
Stability of Glutathione-Forming Enzyme Activity in Gel-A and Gel-B^a

No. of times used	Activity remaining ^b			
	Gel-A		Gel-B	
	-Ac-P	+Ac-P	-Ac-P	+Ac-P
1	0.03	0.15	0.01	0.04
2	0	0.14	0	0
3	0	0.13	0	0
4	0	0.14	0	0

^a The glutathione-producing reactions were repeatedly carried out using Gel-A and Gel-B in the presence or absence of acetyl phosphate. Other reaction conditions are described under Materials and Methods.

^b μmol of glutathione produced/ml/3 h.

^c Ac-P; acetyl phosphate.

genate) $\times 100$) of acetate kinase and glutathione-forming enzyme system after immobilization was about 70 and 80 %, respectively. However, the activities of acetate kinase and glutathione-forming enzyme system in the homogenized toluene-treated immobilized cells were 1125 and 3.31 $\mu\text{mol/g cells/h}$, respectively. From these values, the apparent entrapped activity ((activity of toluene-treated immobilized cells/ activity of homogenized toluene-treated immobilized cells) $\times 100$) was calculated to be about 70 % for both acetate kinase and glutathione-forming enzyme system. These values indicate that the polyacrylamide gel matrix hinders the transport of substrates and/or products to and from the immobilized cells.

Glutathione production by Gel-A and Gel-B

In order to demonstrate the effectiveness of gel-entrapped dextran-bound $\text{N}^6\text{-R}''\text{-ATP}$ in glutathione production, Gel-A and Gel-B were used repeatedly for the reaction (Table III). On repeated use, Gel-B lost the glutathione producing activity and its activity was not restored even in the presence of acetyl phosphate.

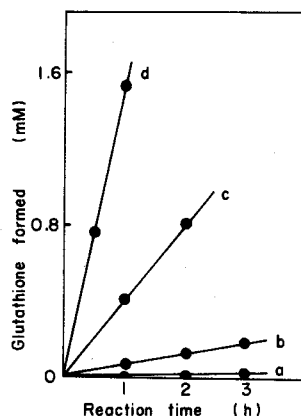


FIG. 2. Time course of glutathione production by Gel-A and Gel-B. The glutathione-producing reactions were carried out with or without ATP in the following systems; (a) Gel-B; (b) Gel-A (c) Gel-A + 5.0 mM ATP; (d) Gel-B + 20 mM ATP. Other reaction conditions were the same as described under Materials and Methods.

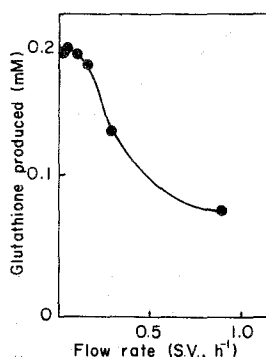


FIG. 3. Effect of flow rates on glutathione production. Gel-A (4.0 ml) was packed in a column (1.0 × 10 cm) and the substrate solution containing 20 mM L-glutamate, 20 mM L-cysteine, 20 mM glycine, 10 mM MgCl₂, 0.1 M Tris-HCl buffer (pH 7.5), and 20 mM acetyl phosphate was passed upward through the column at the flow rates indicated at 37°C, followed by determination of glutathione in the effluent.

However, in the presence of acetyl phosphate, Gel-A showed constant glutathione-producing activity during the period tested. Figure 2 shows the time course of glutathione production by Gel-A and Gel-B in the presence of acetyl phosphate. The glutathione-producing activities of Gel-A and Gel-B were 0.05 and 0.005 $\mu\text{mol/ml gel/h}$, respectively. The activity of Gel-B in the presence of 20 mM ATP was 1.60 $\mu\text{mol/ml-gel/h}$. From these values, the cosubstrate activity of gel-entrapped dextran-bound N⁶-R"-ATP was seen to be only 2.8 % of that of ATP. Addition of 5.0 mM ATP to the reaction mixture containing Gel-A stimulated the rate of glutathione production showing the cosubstrate activity of gel-entrapped dextran-bound N⁶-R"-ATP to be the rate limiting factor in glutathione production by this system.

Continuous production of glutathione by a column packed with Gel-A

Gel-A was packed in a column and substrate solution containing 20 mM L-glutamate, 20 mM L-cysteine, 20 mM glycine, 10 mM MgCl₂, 0.1 M Tris-HCl buffer (pH 7.5) and 20 mM acetyl phosphate was continuously passed through the column in an upward

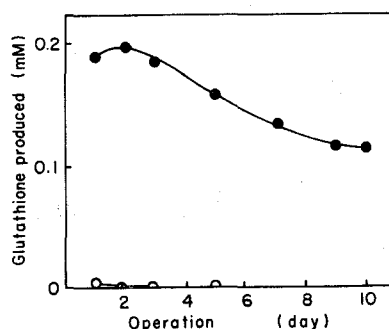


FIG. 4. Continuous production of glutathione. The substrate solution described in the legend to Fig. 3 was passed continuously at $S. V. = 0.1 \text{ h}^{-1}$ at 37°C and glutathione in the effluent was determined. Open circles indicate the results obtained using the same system without acetyl phosphate.

direction. Figure 3 shows the effect of substrate flow rates on glutathione production. The conversion of the three constituent amino acids into glutathione was only about 1.0 % at flow rates below space velocity (SV) = 0.1 h^{-1} at 37°C . Figure 4 shows the deterioration of the glutathione-producing activity of the column operated at 37°C with a flow rate of $SV = 0.1 \text{ h}^{-1}$. The half-life of this column was calculated to be 11 days. In the absence of acetyl phosphate, the activity of the column was lost after operation for only several hours.

DISCUSSION

In construction of a reactor containing enzymes requiring ATP for activity, it is desirable to retain ATP in the reactor, since the expense of a continuous ATP supply represents an economic barrier to the development of efficient processes such as glutathione synthesis involving such enzymes. To retain ATP in a reactor, it was converted into a high molecular weight form by attachment to dextran and the dextran-bound ATP was co-immobilized in a polyacrylamide gel lattice with whole *E. coli* B cells having all of the enzymes responsible for the ATP regeneration

and glutathione synthesis.

However, the glutathione-producing activity of these gels was rather low, probably for the following reasons. First, the cosubstrate activity of dextran-bound ATP is lower than that of ATP itself, though the activity was dependent on the binding number of ATP analogue to the dextran polymer. This low activity of dextran-bound ATP seems to be caused by the shielding and restrictive effects of dextran on the availability and mobility of ATP. The dependence of activity on binding number is probably caused by the crosslinking of dextran that takes place during the activation of the dextran and coupling of the activated polysaccharide with ATP analogues. Second, by entrapping the high molecular weight ATP derivatives within the polyacrylamide gel lattice, steric interferences restricted its availability and mobility. Third, in the reaction system employed, the dextran-bound ATP is involved in the three sequential enzyme reactions catalyzed by acetate kinase and the constituents of the glutathione-forming enzyme system and it is difficult to expect a single form of dextran-bound ATP to show high activity in all three enzyme reactions.

Despite the low activity of gel-entrapped dextran-bound ATP, it functioned in glutathione-producing reactions. This was evident since Gel-A containing dextran-bound ATP did not lose the glutathione-producing activity during the repeated use, while Gel-B did (Table III). The glutathione-producing activity of Gel-A after repeated use was apparent only in the presence of acetyl phosphate. Thus, an ATP regenerating system consisting of dextran-bound ATP and acetate kinase coimmobilized with whole *E. coli* B cells in polyacrylamide gel, can be used to produce

glutathione continuously. Further improvements such as synthesis of ATP analogues with higher cosubstrate activities are likely to make the ATP regeneration system a more efficient one.

SUMMARY

Water-soluble high molecular weight derivatives of ATP were prepared by coupling N⁶-substituted ATP analogues to dextran. These dextran-bound ATP analogues served as cosubstrates for acetate kinase (EC 2.7.2.1) and the two enzymes involved in synthesis of glutathione from its constituent amino acids (glutathione-forming enzyme system: γ -glutamylcysteine synthetase (EC 6.3.2.2) plus glutathione synthetase (EC 6.3.2.3)). To retain these dextran-bound ATP analogues in an enzyme reactor, they were co-immobilized in polyacrylamide gel with whole *Escherichia coli* B cells containing acetate kinase and the glutathione-forming enzyme system. The ability of gel-entrapped dextran-bound ATP analogues to act as cosubstrate for the enzymes involved in glutathione production was investigated in a reactor system.

Section 2. Glutathione production coupled with an ATP regeneration system

INTRODUCTION

For the production of glutathione, the construction of an ATP regeneration system is indispensable for the reaction efficiency and process economy, since this tripeptide is biosynthesized via ATP-requiring reactions catalyzed by γ -glutamylcysteine synthetase and glutathione synthetase.

Thus, I first investigated the utilization of glycolytic pathway in *Saccharomyces cerevisiae* cells as an ATP regeneration system. As a result, glutathione was found to be continuously produced by the column packed with immobilized *S. cerevisiae* cells in polyacrylamide gel, but the maintenance of glycolytic activity was rather difficult.

Subsequently, I investigated the application of dextran-bound ATP to glutathione synthetic processes. By using the column packed with *Escherichia coli* B cells co-immobilized with dextran-bound ATP in polyacrylamide gel, glutathione was also produced continuously, though the amount produced was low.

To establish a more efficient glutathione production system, I have studied the possibility utilizing the acetate kinase reaction as an ATP regeneration system. The basis for the application of acetate kinase reaction for ATP regeneration system was developed by Stramond et al.⁷⁵⁾ to produce gramicidin S. They isolate acetate kinase and gramicidin S synthase from microbial cells by affinity chromatography and immobilized these isolated enzymes on Sepharose 4B. To avoid these intricate and tedious procedures, I directly immobilized whole *E. coli* B cells possessing acetate kinase and glutathione synthesizing activities in carra-

geenan gel and investigated the feasibility of acetate kinase reaction as an ATP regeneration system in glutathione production. In this section, the term of glutathione synthetases represents the combined reaction system of γ -glutamylcysteine synthetase and glutathione synthetase in *Escherichia coli* B.

MATERIALS AND METHODS

Chemicals ATP was purchased from Kojin Co., Ltd., Tokyo, Japan. Acetyl phosphate was purchased from Sigma Chemical Co., St. Louis, MO, USA.

Cultivation and immobilization of *E. coli* B cells The *E. coli* B strain RM-723 isolated as a revertant of cysteine-requiring mutant of *E. coli* B was grown on the nutrient medium (Medium A described in section 1 in Chapter I) with reciprocation for 20 h at 30 °C. After cooling the culture to about 0°C, cells were harvested by centrifugation, washed once with saline and immobilized in carrageenan gel lattice following the procedure given by Takata et al.⁷⁶. In this method, 0.25 g (wet wt.) of cells were immobilized in 1.0 ml of carrageenan gel.

Treatment of immobilized cells with organic solvents or detergents

To enable efficient transport of phosphate esters into cells, the immobilized cells (10 ml) were suspended in 100 ml of 0.5 mM Tris-HCl buffer (pH 7.0) containing 0.5 mM L-cysteine and organic solvents or detergents at the concentrations shown in Table 1. Similarly, intact cells (2.5 g as wet wt.) were also suspended in 5.0 ml of the same solution. The treatments were carried out at 25 °C for 1 h with shaking.

Preparation of cell and immobilized cell homogenates The methods employed for the preparation of cell and immobilized cell homogenates were the same as described in the section 1-3 of Chapter I.

Hardening procedures of immobilized cells To suppress the leakage of enzymes from the gel, immobilized cells were treated with glutaraldehyde(GA) in the presence of hexamethylenediamine(HMDA) as follows. Immobilized cells (8 ml) were suspended in 60 ml of 0.33 M potassium phosphate buffer (pH 7.0) containing 2 % KCl and 0.08 M HMDA. The mixture was incubated with gentle shaking for 10 min at 0 °C. Then, 5 ml of 12.5 % GA solution was added to this mixture and the incubation was continued for 1 h at 0°C. The immobilized cells thus obtained were extensively washed with 2 % KCl containing 0.5 mM L-cysteine (pH 7.0).

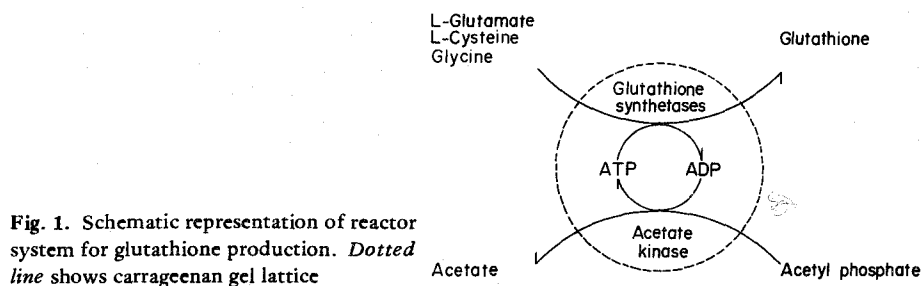
Assay system Glutathione-producing activity was determined by incubating the cells (0.6 g as wet wt.) or immobilized cells

(2.5 ml) in 5.0 ml mixture containing 20 mM L-glutamate, 20 mM L-cysteine, 20 mM glycine, 25 mM $MgCl_2$, 50 mM potassium phosphate buffer (pH 7.0), 20 mM acetyl phosphate and 20 mM ATP at 37 °C for 1 h with shaking. The 0.2 ml of reaction mixture was pipetted out periodically, and the changes in concentrations of glutathione, acetyl phosphate and ATP were determined. Glutathione produced was determined by the method of Tietze⁴⁹. Acetyl phosphate was determined by the method of Lipmann et al.⁷⁴. Acetate kinase and glutathione synthetase activities were determined as described in the section 1 of this Chapter.

RESULTS

Reactor system for glutathione production

The reactor system for glutathione production is shown in Figure 1. This reactor consists of two reaction systems. One is the glutathione synthetic system catalyzed by glutathione synthetases. The other is ATP regeneration system catalyzed by acetate kinase. *E. coli* B(strain RM723) cells contain all the enzymes responsible for the glutathione synthesis and ATP regeneration. The cells were immobilized in carrageenan gel lattice. As shown in Table 1, the apparent glutathione-producing activity of freshly prepared immobilized cells was very low. Thus, for the activation, immobilized cells were treated with organic solvents or detergents. Among the treatment, treatment with toluene markedly increased the glutathione-producing activity.



Glutathione-producing activities of various cell preparations

Table 1. Effect of various agents on glutathione-producing activity of immobilized cells

Treatment with		Glutathione-producing activity ($\mu\text{mol/ml-gel/h}$)
None		2.2
Toluene	(8.0 %)	16.6
Acetone	(100 %)	0
Butanol	(7.1 %)	0
STAC ^a	(0.1 %)	13.2
CTAB ^b	(0.1 %)	3.2
SDS ^c	(0.1 %)	0

a Stearyl trimethyl ammonium chloride

b Cetyl trimethyl ammonium bromide

c Sodium dodecyl sulfate

Immobilized cells (10 ml) were suspended in 100 ml of 5 mM Tris-HCl buffer (pH 7.0) containing 0.5 mM L-cysteine and various agents at the concentrations indicated in Table. Treatment was carried out with shaking for 1 h at 25°C. Glutathione-producing activities were determined as described in Methods and Materials

As can be easily seen in Figure 2, repeated use of the toluene-treated immobilized cells resulted in loss of the glutathione-producing activity. So, the immobilized cells were hardened with GA and HMDA before toluene treatment, since the loss of glutathione-producing activity was thought to be caused by the leakage of enzymes out of gel. Table 2 summarizes the glutathione-producing activities of various cell preparations. The hardening processes of immobilized cells did not improve the glutathione-producing activity. Therefore, to attain the maximum glutathione-producing activity of cell and immobilized cell

merizes the glutathione-producing activities of various cell preparations. The hardening processes of immobilized cells did not improve the glutathione-producing activity. Therefore, to attain the maximum glutathione-producing activity of cell and immobilized cell

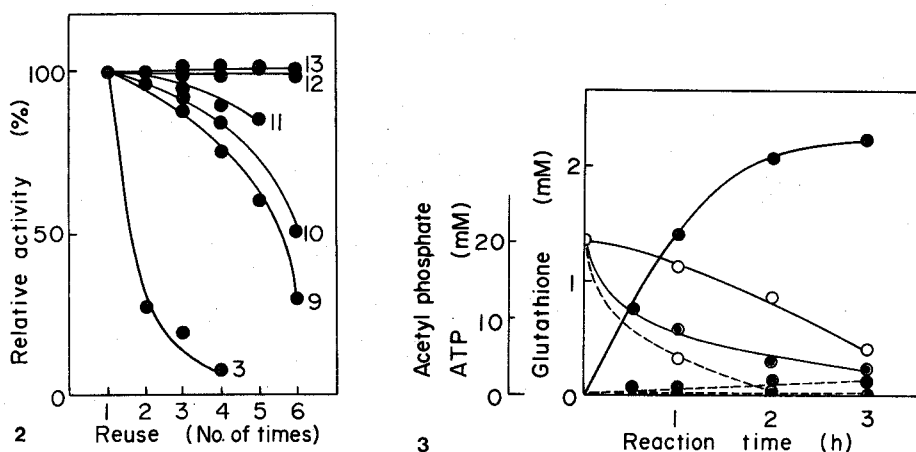


Fig. 2. Reuse of intact cells and immobilized cells for glutathione-producing reactions. Enzyme activities were determined as described in Methods and Materials. The intact cells and immobilized cells were thoroughly washed with 0.85% saline solution containing 0.5 mM L-cysteine (pH 7.0) each time before the addition of a new substrate solution. Curve No. in figure indicates the cell preparation No. listed in Table 2. In figure 100% activity was quoted relative to the initial activity given in Table 2

Fig. 3. Effect of acetyl phosphate on glutathione-producing activity of immobilized cells. Glutathione-producing reactions were carried out with (—) or without (-----) acetyl phosphate. Other reaction conditions were described in Methods and Materials. Symbols: (●): glutathione; (○) ATP; (⊙): acetyl phosphate

Table 2. Enzyme activities of various cell preparations

No. Cell preparations	Glutathione-producing activity ($\mu\text{mol/g-cells/h}$)	Relative degrading activity ^c of	
		ATP	Acetyl phosphate
1. Intact cells	2.2	-	-
2. Homogenate of cells	33.7	-	-
3. Toluene-treated cells	33.3	100	100
4. Immobilized cells	2.6	-	-
5. Immobilized cells hardened with GA ^a (10 min) ^b	1.3	-	-
6. Immobilized cells hardened with GA and HMDA ^c (10 min)	2.6	-	-
7. Immobilized cells hardened with GA and HMDA (20 min)	2.8	-	-
8. Immobilized cells hardened with GA and HMDA (60 min)	2.7	18	22
9. Toluene-treated immobilized cells	16.6	-	-
10. Toluene-treated immobilized cells hardened with GA (10 min)	5.2	-	-
11. Toluene-treated immobilized cells hardened with GA and HMDA (10 min)	15.8	-	-
12. Toluene-treated immobilized cells hardened with GA and HMDA (20 min)	16.4	-	-
13. Toluene-treated immobilized cells hardened with GA and HMDA (60 min)	16.6	10 ^d	80 ^d
14. Homogenate of toluene-treated immobilized cells hardened with GA and HMDA (60 min)	25.9	88	92

^a Glutaraldehyde

^b Hardened time

^c Hexamethylenediamine

^d Activity after repeated use for five times for glutathione-producing reactions (see Fig. 2)

^e All values are quoted relative to the activity of toluene-treated cells. Enzyme activities were determined as described in Methods and Materials using various cell preparations

preparations, toluene treatment was indispensably required. The toluene-treated immobilized cells hardened with GA in the presence of HMDA (cell preparation No. 11, 12, and 13) showed the higher glutathione producing activities than other immobilized cells, and the yield of glutathione-producing activity was about 50 % (activity of cell preparation Nos. 11, 12 or 13/activity of cell preparation No. 2).

Reuse of immobilized cells for glutathione-producing reaction

Figure 2 shows the glutathione-producing activity of immobilized cells after repeated use for glutathione-producing reactions. Though the toluene-treated immobilized cells lost the glutathione-producing activity rapidly, the immobilized cells hardened with GA

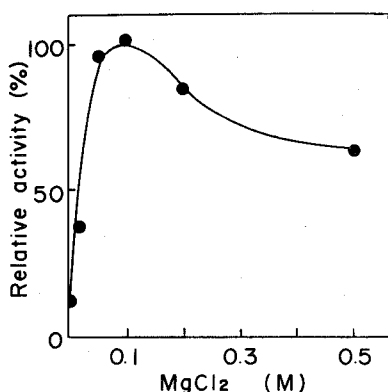


Fig. 4. Effect of MgCl_2 concentration on glutathione-producing activity of immobilized cells. Reaction was carried out as described in Methods and Materials except of MgCl_2 concentrations. To $7.6 \mu\text{mol}$ of glutathione produced/ml-gel/h 100% glutathione-producing activity was equal

or GA and HMDA before toluene treatment were able to be used several times for glutathione producing reactions. Especially marked stabilization of glutathione-producing

activity was observed when the immobilized cells were treated for over 20 min with GA in the presence of HMDA. In the following experiments, unless otherwise given, immobilized cells represent the toluene-treated immobilized cells hardened with GA for 1 h in the presence of HMDA. Similarly, intact cells represent the toluene-treated cells.

Time course of glutathione production

Figure 3 shows the time course of glutathione production by immobilized cells in the presence or absence of acetyl phosphate. Only in the presence of acetyl phosphate, was glutathione efficiently produced at the rate of $2.6 \mu\text{mol/h/ml-gel}$. MgCl_2 concentration in reaction mixture greatly influenced upon the glutathione-producing activity of immobilized cells. Maximum effect was attained in the presence of 0.05-0.1 M MgCl_2 (Fig. 4).

Properties of immobilized cells

1) *pH effect* The effect of pH on acetate kinase, glutathione synthetase and glutathione-producing (i.e. coupled reaction system of acetate kinase and glutathione synthetases) activities is shown in Figure 5. The optimal pH for glutathione producing ac-

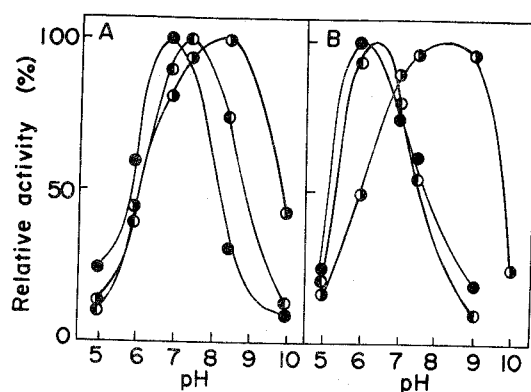


Fig. 5A and B. Effect of pH on enzyme activities in intact cells (A) and in immobilized cells (B). Enzyme activities were determined under various pH. Buffers used were sodium acetate for pH 5.0 and 6.0, potassium phosphate for pH 7.0 and 7.5, and borate-NaOH for pH 8.5 to 10. All buffers were used at 0.025 M. For intact cells (A), 100% activity of acetate kinase, glutathione synthetase and glutathione-producing system correspond to 2.1 mmol of acetyl phosphate formed/g-cells/h, 1.3 μ mol of glutathione formed/g-cells/h and 33.3 μ mol of glutathione formed/g-cells/h, respectively. For immobilized cells (B), 100% activity of acetate kinase, glutathione synthetase and glutathione-producing system correspond to 1.1 mmol of acetyl phosphate formed/ml-gel/h, 0.6 μ mol of glutathione formed/ml-gel/h and 16.6 μ mol of glutathione formed/ml-gel/h, respectively. Symbols: (○): acetate kinase activity; (◐): glutathione synthetase activity; (●): glutathione-producing activity

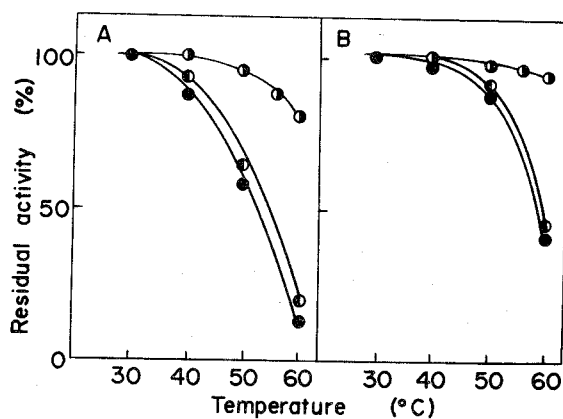


Fig. 6A and B. Effect of temperature on enzyme stabilities in intact cells (A) and in immobilized cells (B). Intact cells (0.5 g as wet wt.) or immobilized cells (1.0 ml) were suspended in 2.0 ml of 5.0 mM Tris-HCl buffer (pH 7.0) and heated for 10 min at the indicated temperature. Residual enzyme activities were assayed as described in Methods and Materials. Symbols: (○): acetate kinase activity; (◐): glutathione synthetase activity; (●): glutathione-producing activity

tivity of immobilized cells was 6.0, a slightly lower than that of intact cells.

2) *Heat stability* Heat stability of glutathione-producing activity was shown in Figure 6. By immobilizing with carrageenan gel, this activity is better protected from the thermal denaturation at higher temperature.

Table 3. Effect of various agents on acetate kinase activity and degrading activities of acetyl phosphate and ATP

Addition		Relative activity of		
		Acetate kinase	Acetyl phosphate-degrading enzymes	ATP-degrading enzymes
None		100 (460) ^a	100 (22.5) ^b	100 (25.8) ^c
Inorganic phosphate	100 mM	97.5	92.5	63.5
	200 mM	80.5	86.4	52.1
Pyrophosphate	10 mM	24.3	19.6	74.5
	25 mM	13.6	16.2	66.7
	50 mM	2.2	1.2	48.9
NaF	50 mM	77.1	75.1	70.5
	100 mM	68.9	63.1	63.3
L-thyroxine	1.0 mM	98.1	97.8	97.6

Enzyme activities were determined as described in Methods and Materials with or without various agents shown above. All values are quoted relative to the activity of without addition as 100

(): Specific activity

^a μmol of acetyl phosphate formed/ml-gel/h

^b μmol of acetyl phosphate degraded/ml-gel/h

^c μmol of ATP degraded/ml-gel/h

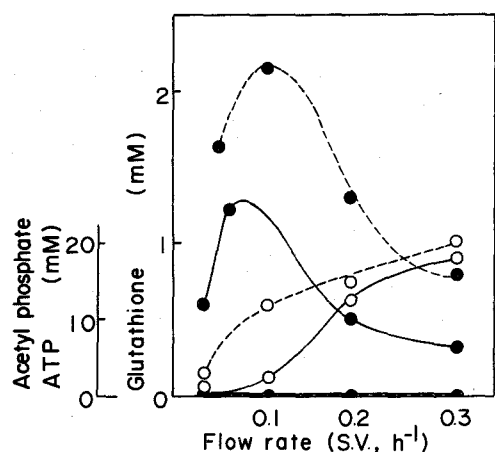


Fig. 7. Effect of flow rates on glutathione production. Five milliliter of immobilized cells were packed in column (1.0 cm x 10 cm) and substrate solution was passed upward through this column at the various flow rates at 37°C. Substrate solution was passed by two different methods, I (—) and II (---). Method I: substrate solution of 20 mM L-glutamate, 20 mM L-cysteine, 20 mM glycine, 25 mM MgCl₂, 50 mM potassium phosphate buffer (pH 7.0), 20 mM ATP, and 20 mM acetyl phosphate was supplied directly. Method II: twofold concentrated acetyl phosphate solution and the mixture of other components were prepared separately. These two solutions were passed simultaneously at the same flow rate and mixed them at the entrance of column. Glutathione (●), residual ATP (●), and residual acetyl phosphate (○) in effluent were determined

ATP- and acetyl phosphate-degrading activities As shown in Figure 3, added ATP to reaction mixture was gradually degraded by immobilized cells even in the presence of acetyl phosphate. However, most of this ATP-degrading activity was destroyed during the repeated use for five times (Table 2). On the other hand, decrease in acetyl phosphate-degrading activity by the repeated use was only 20 % of initial activity. To inhibit the acetyl phosphate-degrading activity, various agents were added to the reaction mixture (Table 3). The agents inhibiting the degrading

activity of acetyl phosphate simultaneously inhibited the acetate kinase activity.

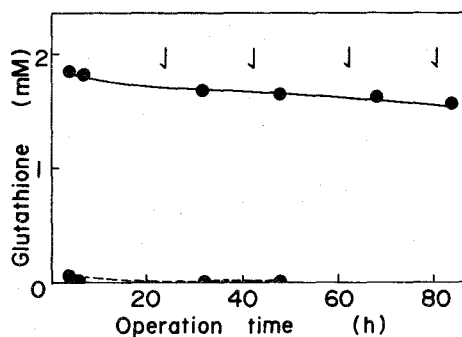
Continuous production of glutathione

1) *Effect of flow rate* To produce glutathione continuously, immobilized cells were packed in a column and the substrate solution was passed at the various flow rates by methods, I and II. Method I is a direct supply of complete substrate solution from the bottom of column. In method II, acetyl phosphate and other substrate solution were separately prepared and these two solutions were simultaneously supplied by mixing them at the entrance of column. As can be seen in Figure 7, supply of substrate by Method II was more effective for glutathione production and by this method, 2.3 mM of glutathione was produced at the space velocity (SV) = 0.1 h^{-1} at 37°C .

2) Operational stability of glutathione-producing activity

To know the feasibility of this glutathione production system, the operational stability of glutathione-producing activity was investigated. The substrate solution was continuously supplied by the Method II at $\text{SV} = 0.1 \text{ h}^{-1}$ at 37°C and glutathione in effluent was determined. As shown in Figure 8, only in the presence of acetyl phosphate, glutathione was continuously produced,

Fig. 8. Operational stability of immobilized *E. coli* cell column. Operational conditions were the same as described in legend to Fig. 7. Substrate solution was passed by the method II at $\text{S.V.} = 0.1 \text{ h}^{-1}$ at 37°C . Dotted line shows the parallel run of the same column without acetyl phosphate. Acetyl phosphate solution was changed into freshly prepared one at the 20 h intervals (indicated by arrow)



and the half-life of this column was 8 days. During the continuous operation, freshly prepared acetyl phosphate solution was introduced at about 20 h intervals, since acetyl phosphate was degraded in the aqueous condition.

DISCUSSION

For the optimal production of glutathione, an efficient ATP regeneration system is indispensable, since ADP is produced

Table 4. Summary of glutathione-producing activities of immobilized microbial cells containing ATP regeneration system

Carrier	Cells immobilized	ATP regeneration system	Glutathione-producing activity ¹	Operational stability ²
Polyacrylamide gel	<i>S. cerevisiae</i>	Glycolysis	0.65	32 (30°C)
Polyacrylamide gel	<i>E. coli</i> B <i>S. cerevisiae</i>	Glycolysis Adenosine kinase Adenylate kinase	0.86	22 (30°C)
Polyacrylamide gel	<i>E. coli</i> B	Acetate kinase	0.10	11 (37°C)
Carrageenan gel	<i>E. coli</i> B	Acetate kinase	2.60	8 (37°C)

1. μ mole of glutathione produced/h/ml-gel

2. Half-life, day

not only by glutathione synthetic reaction, but also by the action of ATP-degrading activity in immobilized cells and it strongly inhibits the glutathione synthetase reaction⁵⁶). To avoid this inhibition by ADP and to maintain ATP concentration at a high level, I utilized the acetate kinase reaction in *E. coli* B cells as an ATP regeneration system and investigated the feasibility of this system in glutathione production.

As can be seen in Figure 3, by adding acetyl phosphate to reaction mixture, efficient glutathione production was attained

by using immobilized *E. coli* B cells. The most troublesome factor of this glutathione production system was the degradation of expensive acetyl phosphate, possibly, as a result of phosphatases or acetyl phosphatase in immobilized cells. Therefore, the methods were sought to decrease the rate of degradation of acetyl phosphate. However, treatments of immobilized cells with organic solvents, detergents, heat and acid were all ineffective in decreasing the degrading activity of acetyl phosphate without damaging the enzymes responsible for the glutathione production (data are not shown). I also tested the effect of phosphatase inhibitors or several metal ions. As shown in Table 3, pyrophosphate strongly inhibited the degrading activity of acetyl phosphate, but it also inhibited the acetate kinase activity. L-Thyroxine, a specific inhibitor to acetyl phosphatase of mammalian tissues⁷, was also not effective. On the other hand, various metal ions accelerated the degradation of acetyl phosphate (data not shown).

For industrial application, the operational stability of glutathione-producing activity is very important. By treatment with glutaraldehyde and hexamethylenediamine, the immobilized cells possessed superior properties on both the activity and operational stability (Table 2). These properties may be due to the reduction of mobility of enzymes responsible for glutathione production in the carrageenan gel lattice and/or suppression of leakage of these enzymes from the gel by crosslinking among glutaraldehyde, hexamethylenediamine, cell walls and some of the cell proteins.

In comparison with toluene-treated cells, the glutathione-producing activity of hardened immobilized cells was rather low (Table 2). This was possibly due to the partial inactivation of enzymes exposed to hardening reagents and diffusion barrier of

carrageenan gel lattice for the transport of substrate and/or products.

Table 4 summarizes the glutathione-producing activities of immobilized microbial cells containing an ATP regeneration system. Of the glutathione production systems examined, the system developed here showed the highest glutathione-producing activity, though the operational stability was slightly inferior to that of immobilized *Saccharomyces cerevisiae* cells with polyacrylamide gel. Therefore, by improvement of the operational stability and by effective suppression of degrading activity of acetyl phosphate, the glutathione production system studied here appears to be the more promising one.

SUMMARY

Escherichia coli B cells possessing glutathione synthetase and acetate kinase activities were immobilized with carrageenan gel. To enhance the operational stability, immobilized cells were treated with hardening agent, glutaraldehyde in the presence of hexamethylenediamine. The continuous production of glutathione was investigated using the column packed with immobilized *E. coli* B cell preparations. Glutathione was continuously produced by this column in the presence of acetyl phosphate and the half-life of this column was calculated to be 8 days at the flow rate of $SV=0.1 \text{ h}^{-1}$ at 37°C .

CHAPTER IV

Construction of *Escherichia coli B* strains having high glutathione-synthesizing activity

In the previous Chapter, the properties of various reactor systems containing an ATP regeneration process were investigated in detail to produce glutathione efficiently and economically. Among the reactor systems examined, the immobilized *E. coli B* cell system showed the highest glutathione-producing activity, but its operational stability was rather low in addition to the difficulty of expensive supply of acetyl phosphate. So, the coupled reactor system of *Saccharomyces cerevisiae* cells with *E. coli B* cells was found to be most suitable on both the high glutathione producing-activity and good operational stability. In this reactor system, *S. cerevisiae* cells catalyzed the conversion of adenosine to ATP consuming glucose by glycolytic pathway. On the other hand, *E. coli B* cells carried out the glutathione production from its constituent amino acids using ATP generated from adenosine.

To further improve the productivity of this reactor system, the construction of *Escherichia coli B* strains having high glutathione-producing activity was investigated by biochemical and gene engineering technichs. This Chapter deals with these results.

Section 1. Excretion of glutathione by methylglyoxal-resistant *Escherichia coli* B

INTRODUCTION

Methylglyoxal (MG) is toxic metabolite for *Escherichia coli* at millimolar concentrations as shown by Ackerman et al.⁸⁶⁾, Freedberg et al.⁸⁷⁾ and by Krymkiewicz et al.⁸⁸⁾. It is synthesized via a non-phosphorylated pathway from dihydroxyacetone phosphate⁸⁹⁾. This pathway is considered to be finely regulated and regulation mechanisms have been studied in detail by Hopper & Cooper⁹⁰⁾, and by Freedberg et al.⁸⁷⁾. Hopper & Cooper⁹⁰⁾ showed the activity of MG synthase was regulated by inorganic phosphate. Freedberg et al.⁸⁷⁾ also showed that the increased capacity for MG metabolism in MG-resistant cells was due to the elevated activity of the glyoxalase system, which requires the participation of glutathione for activity.

However, no report on the regulation of activity of the glyoxalase system has described the change in the concentration of glutathione in bacteria and the change in activity of the glutathione-forming enzyme system. To clarify the mechanism for MG resistance further, I have investigated the relationship between activities of enzymes involved in MG metabolism and glutathione synthesis in MG-resistant cells of *Escherichia coli* B.

METHODS AND MATERIALS

Chemicals Methylglyoxal (MG) and dihydroxyacetone phosphate (DHAP) were purchased from Sigma Chemical. Co., St. Louis MO.

Microorganisms used Microorganisms used and their properties are listed in Table 1.

Isolation of an MG-resistant mutant (RM-9-10) Strain RM-7-23

was treated with NTG by the method of Adelberg et al.⁹¹ and spread on agar Davis-Mingioli minimal medium¹⁰⁰ containing 1.4 mM MG. After 7 days at 30 °C, the largest colony formed was transferred to 2.0 ml of liquid minimal medium containing 1.4 mM MG. Cultivation was carried out with reciprocal shaking at 30 °C for 16 h and then samples of appropriately diluted culture were spread on agar minimal medium containing 1.4 mM MG. After 4 day at 30 °C, the largest colony formed was selected as an MG-resistant mutant and designated RM-9-10.

Assay of glyoxalase system Cells growing exponentially on 150 ml Davis-mingioli minimal medium at 30 °C with shaking were harvested, washed once with 5.0 mM potassium phosphate buffer (pH 6.6) and suspended in the same buffer. This cell suspension was disrupted ultrasonically at 90 KHz for 5 min and then centrifuged at 25,000 xg for 30 min. The resultant supernatant was dialyzed against the same buffer overnight. For determination of the activity of the glyoxalase system (glyoxalase I (EC 4.4.1.5) and glyoxalase II (EC 3.1.2.6)), a reaction mixture (1.0 ml) containing 0.5 mM MG, 50 mM potassium phosphate buffer (pH 6.6) and dialyzed extract (0.1-0.5 mg/ml protein) was incubated at 30 °C for 1 h and then the residual MG was determined colorimetrically by the method of Hopper & Cooper⁹⁰.

Assay of the glutathione-forming enzyme system Dialyzed extracts were prepared as described above except that the buffer used contained 0.5 mM L-cysteine. The activity of the glutathione-forming enzyme system (γ -glutamylcysteine synthetase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3)) was assayed by coupling with the ATP regeneration reaction catalyzed by acetate kinase as described in the section 1 of Chapter III. Glutathione formed was determined by the method of Tietze⁴⁹.

Assay of MG synthase Dialyzed extracts were prepared as described above except that imidazole/HCl buffer (pH 7.0) was used instead of phosphate buffer. For the determination of MG synthase activity (EC 4.2.99.11), a reaction mixture (1.0 ml) containing 0.5 mM DHAP, 50 mM imidazole/HCl buffer (pH 7.0) and dialyzed extract (0.1-0.5 mg/ml protein) was incubated at 30°C for 1 h, and then the MG formed was determined colorimetrically by the method of Hopper & Cooper⁹⁰. In all cases, protein was determined by the Lowry method²⁶.

Determination of glutathione Cultures were cooled and centrifuged at 3,000 rev./min for 10 min. Extracellular glutathione was determined in this supernatant by the method of Tietze⁴⁹. For the determination of intracellular glutathione, cells were washed once with chilled 0.85 % saline solution and resuspended in water to give 10 mg (wet wt.)/ml; 0.5 ml was then heated at 100 °C for 1 min, immediately cooled, centrifuged at 3,000 rev./min for 10 min and glutathione in supernatant was determined by the method of Tietze⁴⁹.

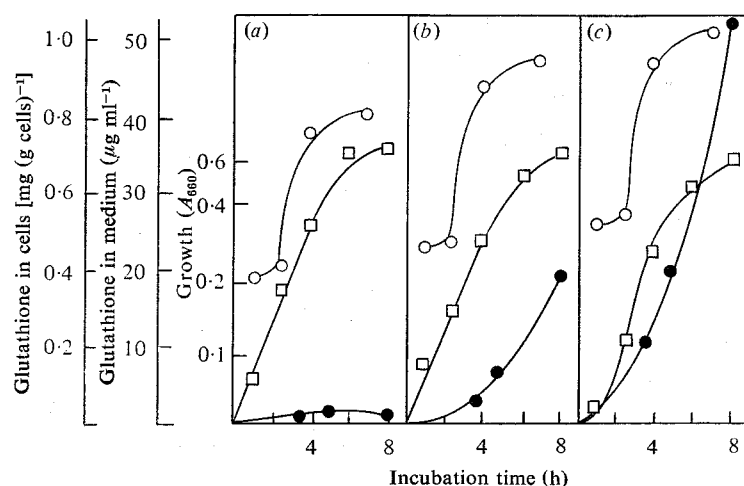


Fig. 1. Excretion of glutathione into the medium by wild-type *Escherichia coli* strain 355 and MG-resistant strain RM-9-10. The wild-type strain was grown in DM medium in the absence of MG (a). The MG-resistant strain was grown in DM medium in the absence (b) or presence (c) of 1.4 mM-MG. Growth was followed turbidimetrically at 660 nm. ●, Glutathione in medium; ○, glutathione in cells; □, growth.

Table 1. Activities of enzymes involved in MG and glutathione metabolism

Enzyme activities were determined using extracts prepared from cells growing exponentially on DM medium with the addition indicated. Assay conditions are described in Methods.

Strain	Properties	Addition to DM medium	MG synthase*	Glyoxalase system†		Glutathione-forming system‡
				-GSH	+GSH	
355	Wild-type	None	0.28	0.031	0.072	0.13
M-7	Cysteine auxotroph	L-Cysteine, 0.5 mM	0.27	0.030	0.068	0.12
RM-7-23	Revertant of M-7	None	0.25	0.030	0.061	0.11
RM-9-10	Resistant to 1.4 mM-MG	None	0.29	0.042	0.140	0.19
RM-9-10	Resistant to 1.4 mM-MG	MG, 1.4 mM	0.31	0.045	0.160	0.26

* Expressed as $\mu\text{mol MG formed min}^{-1} (\text{mg protein})^{-1}$.

† Expressed as $\mu\text{mol MG degraded min}^{-1} (\text{mg protein})^{-1}$; activities were assayed in the presence or absence of 1.0 mM-glutathione (GSH).

‡ Expressed as $\mu\text{mol glutathione formed h}^{-1} (\text{mg protein})^{-1}$.

RESULTS AND DISCUSSION

Under normal conditions most microorganisms excrete little glutathione from the cells. However, in the course of a study on glutathione metabolism using a revertant (RM723) of a cysteine auxotroph, I found that an MG-resistant strain (RM-9-10) derived from strain RM723 excreted glutathione into the medium, though the parent strain did not (Fig. 1). In addition to the excretion of glutathione, the MG-resistant cells accumulated intracellular glutathione to considerably higher concentrations than did the parent strain. This excretion and accumulation of glutathione

was further accelerated during growth on MG-containing medium. In the presence of MG, the concentration of glutathione excreted into the medium was about 50-fold higher than in the wild-type culture after 8 h (Fig. 1).

The reason for this glutathione overproduction was found to be that the activity of glutathione-forming enzyme system (a combined enzyme system of γ -glutamylcysteine synthetase and glutathione synthetase) in the MG-resistant strain was much higher than in the parent strain. Furthermore, the activity of the glyoxalase system was also higher in MG-resistant cells (Table 1). Thus, it appears that MG resistance was caused by a simultaneous increase in glutathione synthetic activity and in MG disposal activity. As originally suggested by Freedberg et al.⁸⁷⁾, MG-resistance was acquired through the increased capacity to remove MG rather than a simple tolerance to MG.

Thus, MG-resistant *E. coli* overproduced glutathione and excreted it, especially during the growth on MG-containing medium. This excretion of glutathione was presumably due to the change in membrane structure or membrane components caused by contact with MG, since MG is thought to cause a drastic change in cell membranes⁹²⁾.

SUMMARY

A methylglyoxal-resistant mutant of *Escherichia coli* B excreted glutathione into growth medium, especially during the growth on medium containing methylglyoxal. In the presence of methylglyoxal, the total amount of glutathione excreted was increased about 50 fold over that of the wild-type strain. The resistant mutant had high activities of two enzyme systems: a glutathione-forming enzyme system and glyoxalase system. Methyl-

glyoxal resistance appeared to be due to the simultaneous increase in the activities of these two enzyme systems.

Section 2. Isolation of *Escherichia coli* B mutants deficient in glutathione-degrading activity

INTRODUCTION

As shown in previous section, two-fold increase in glutathione-producing activity was found in methylglyoxal-resistant mutant of *E. coli* B. In addition to this increase in activity this mutant could excrete glutathione into growth medium, especially in the presence of methylglyoxal. These properties of methylglyoxal-resistant mutant were advantageous for the efficient production of glutathione.

However, contrary to these advantages, *E. coli* B could degrade glutathione into constituent three amino acids. This was confirmed by the fact that a cysteine auxotroph derived from *E. coli* B could grow on a minimal medium containing glutathione as a sole source of cysteine.

The degradation of glutathione was disadvantageous for the accumulation of large amount of glutathione. To overcome this barrier, the isolation of a mutant deficient in glutathione-degrading activity was investigated. This section deals with this result.

METHODS AND MATERIALS

Chemicals L-γ-glutamyl-p-nitroanilide was purchased from Sigma Chemical Co., St. Louis. MO.

Strains used Two kinds of cysteine auxotrophs were used. M71 was derived from *E. coli* B 355 by NTG treatment by the method of Adelberg et al.⁹¹. M71-1 was also derived from M71 by NTG treatment, but this strain was partially deficient in glutathione-degrading activity.

Isolation of M71-1 Cells of M71 growing exponentially on Davis-Mingioli minimal medium (DM-medium)¹⁰⁰ supplemented with 1 mM

L-cysteine were harvested, washed once with 0.85 % saline solution and then resuspended in 2.5 ml of saline solution to give 10^8 cells/ml. To this cell suspension, 0.5 ml of 1 mg/ml NTG solution was added. After incubation at 30 °C for 30 min with shaking, cells were harvested, washed twice with saline solution and then incubated in the nutrient medium comprising of 1 % yeast extract, 1 % peptone, 0.5 % NaCl and 0.1 % glucose (pH 7.2) at 37 °C for about two generations. After harvesting and washing, the cells were spread on DM medium containing 1 mM glutathione and 10^{-5} M L-cysteine. Plates were incubated at 37 °C for 2 days, and small colonies appeared were selected as mutants deficient in glutathione-degrading activity. One of them was designated M7l-1.

Assay for glutathione-degrading activity Cells growing exponentially in DM-medium supplemented with 1 mM L-cysteine were harvested, washed once with 0.85 % saline solution and then resuspended in 1 ml of 50 mM Tris-HCl buffer (pH 7.0) to make 20 mg(wet wt.)/ml cell concentration. To this cell suspension, 0.1 ml of toluene was added and the mixture was incubated at 30 °C for 30 min with shaking. Glutathione-degrading activity was assayed in the mixture (1.0 ml) containing 2 mM glutathione, 50 mM Tris-HCl buffer (pH 7.0) and 10 mg/ml toluenized cells at 37 °C for 7 h with shaking. The residual glutathione in reaction mixture was determined by the method of Tietze⁴⁹.

Assay for γ -glutamyltranspeptidase activity Cell free extracts were prepared as described in section 1 of this Chapter. The determination of γ -glutamyltranspeptidase activity was performed by adding 0.1 ml of a solution containing the enzyme (0.2-0.5 mg/ml protein) to 0.9 ml of a solution containing L- γ -glutamyl-p-nitroanilide (5 μ moles), $MgCl_2$ (10 μ moles) and Tris-HCl buffer (pH 9.0, 100 mM). After 15 min of incubation at 37 °C, the reaction was terminated by addition of 2 ml of 1.5 N acetic acid and the absorbance was measured at 410 nm. The quantity of p-nitroanilide liberated was calculated from a standard curve (molar extinction:8800).

RESULTS AND DISCUSSION

E. coli B cells degraded glutathione, since the cysteine auxotroph could grow on the minimal

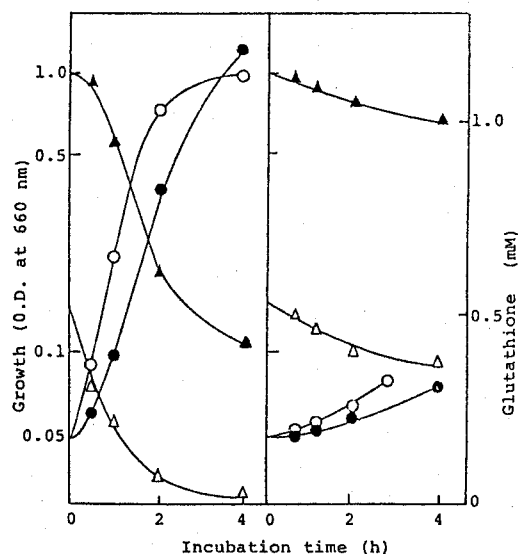


Fig. 1. Degradation of glutathione by cysteine auxotroph M7l (left) and M7l-1 (right) derived from *E. coli* B. Two kinds of cysteine auxotrophs were grown on DM-medium containing glutathione (0.5 mM, open symbol; 1.0 mM, closed symbol) as a sole source of cysteine. (o), Growth; (Δ), glutathione in medium

medium containing glutathione as a sole source of cysteine (Fig. 1).

This result indicated that the two peptide bonds in glutathione was degraded to give three constituent

Table 1. Degradation of glutathione by cysteine auxotrophs derived from *E. coli* B

Strain	Addition (mM)	Glutathione found (mM) after 7h ¹	γ-GTP activity (μmole/h/mg-cells)
M71 (<i>cys</i> ⁻)	None	1.25	12.4
	L-Glu(15), L-Cys (15), Gly (15)	1.05	
	L-Ser(20), Borate(20)	1.67	
M71-1 (<i>cys</i> ⁻)	None	1.88	3.76
	L-Glu(15), L-Cys (15), Gly (15)	1.84	
	L-Ser(20), Borate(20)	1.93	

1) Glutathione(2 mM) was incubated with 10 mg/ml cells

amino acids. So, the methods were sought to obtain mutants having no or low glutathione-degrading activity. It was considered that such strains may be obtained as a cysteine auxotrophs which can not grow on minimal medium containing glutathione as a sole source of cysteine. Thus, the cysteine auxotroph M71 was mutated with NTG and spread on DM-medium containing 1 mM glutathione and 10⁻⁵ M L-cysteine. Large and small colonies were appeared on the plates after 2 days incubation. Large colonies were thought to be cysteine auxotrophs having high glutathione-degrading activity or revertants of cysteine auxotrophs, which no longer require L-cysteine as growth factor. Small colonies were picked up and tested for their ability to degrade glutathione by the method as described in Materials and Methods. Of the 50 colonies tested, one (M71-1) showed the decreased level of activity of glutathione degradation (Table 1). The degradation of glutathione by M71-1 was also little even in the presence of amino acids required for the glutathione synthesis (Table 1). Furthermore, this mutant strain could not grow on the medium containing glutathione as a sole source of L-cysteine (Fig. 1).

To clarify the reason for this low glutathione-degrading activity of M71-1, γ -glutamyltranspeptidase activity was determined and compared with that of M-71, since glutathione-degrading activity in M71 was suppressed in the presence of borate and L-serine, potent inhibitor of γ -glutamyltranspeptidase (Table 1). As a result, this enzyme activity in M71-1 was found to be far lower than that of M71. This result indicated that γ -glutamyltranspeptidase may play an important role in glutathione degradation in *E. coli* B.

SUMMARY

The isolation of mutant strains which can not degrade glutathione was investigated. Such mutants were selected as cysteine auxotrophs which could not grow on the medium containing glutathione as a sole source of L-cysteine. The γ -glutamyltranspeptidase activity in isolated mutant was about $\frac{1}{4}$ of that of parental strain. It was suggested that this enzyme might play an important role in glutathione metabolism in *E. coli* B.

Section 3. Purification and characterization of γ -glutamylcysteine synthetase of *Escherichia coli B*

INTRODUCTION

Glutathione, a tripeptide consisting of L-glutamate, L-cysteine and glycine, is synthesized by the sequential enzyme reactions catalyzed by γ -glutamylcysteine synthetase and glutathione synthetase. γ -Glutamylcysteine synthetase has been isolated from Hog liver⁷⁸⁾, Wheat germ⁷⁹⁾ and bovine lens⁸⁰⁾, and the enzymatic properties of γ -glutamylcysteine synthetase from these sources have been investigated in detail.

However, there is no information on the properties of this enzyme in bacteria, though glutathione plays an important role in bacterial cells. So, the γ -glutamylcysteine synthetase of *E. coli B* was purified and the properties of this enzyme were investigated in detail to elucidate the regulation mechanism in glutathione biosynthetic pathway.

This section deals with these results.

MATERIALS AND METHODS

Chemicals All the chemicals used were purchased from Sigma Chemical Co., St. Louis. MO.

Cultivation *Escherichia coli B* (355) was grown on L-broth comprising of 1.0 % polypeptone, 1.0 % yeast extract, 0.5 % NaCl and 0.1 % glucose (pH 7.2). Cultivation was carried out in 500 ml of medium in a 2 L Sakaguchi flask at 37 °C for 24 h with reciprocal shaking.

Enzyme assay γ -Glutamylcysteine synthetase activity was determined by the method of Jackson⁸¹.

Purification of γ -glutamylcysteine synthetase Unless otherwise noted, all the procedures were carried out at 4 °C and the buffer was 5.0 mM potassium phosphate buffer (pH 7.5). Centrifugation was also carried out at 25,000 xg for 20 min.

1) *Cell extracts* *E. coli* B 355 cells grown on nutrient medium were harvested, washed once with 0.85 % saline solution and then resuspended in buffer. This cell suspension (120 g wet wt. cells, 300 ml) was sonicated at 90 kHz for 25 min. After centrifugation, the supernatant was dialyzed against buffer overnight.

2) *1st ammonium sulfate* To the dialysate (8,420 mg protein, 370 ml), 90 g of solid ammonium sulfate was added. The mixture was stirred for 30 min and then centrifuged. 49 g solid ammonium sulfate was added to the clear supernatant and the mixture stirred for 30 min. The precipitate was collected and dissolved in 100 ml of buffer and dialyzed overnight against buffer.

3) *2nd ammonium sulfate* To the fraction of 1st ammonium sulfate (3140 mg protein, 130 ml), 30 g of solid ammonium sulfate was added and the mixture was stirred for 30 min. The precipitate was discarded after centrifugation. 17 g of solid ammonium sulfate was added to the supernatant obtained and the mixture was stirred for 30 min. Precipitate was collected and dissolved in 50 ml of buffer and dialyzed overnight against buffer.

4) *DEAE cellulose column chromatography* The 2nd ammonium sulfate fraction (774 mg protein, 58 ml) was applied to DEAE cellulose column (5 x 60 cm) equilibrated with buffer. The adsorbed proteins were eluted (10 ml/tube/6 min) with linear gradient of buffer containing KCl over the concentration range of 0 to 0.5 M. The active fractions (Fr. No. 65-100), which were eluted with about 0.3 M of KCl, were collected, concentrated with a Amicon UM-10 membrane to about 50 ml and dialyzed overnight against buffer.

5) *Heat treatment* The dialysate (91.3 mg protein, 52 ml) was heated to 50°C for 20 min. The precipitated materials were discarded after centrifugation. The supernatant was applied to hydroxylapatite column.

6) *Hydroxylapatite column chromatography* The enzyme solution recovered after heat treatment (7.7 mg protein, 48 ml) was applied to a hydroxylapatite column (4 x 35 cm) equilibrated with buffer. The adsorbed proteins were eluted with a linear gradient of potassium phosphate buffer (pH 7.5) from 5.0 to 300 mM. Fractions were collected as 1.5 ml/tube/6 min. The active fractions were eluted with about 0.08 M of buffer (Fr. No. 25-50), collected and concentrated to 25 ml with Amicon UM-10 membrane.

7) *3rd ammonium sulfate* To the eluate (2.41 mg protein, 25 ml) 2 g of solid ammonium sulfate was added and the mixture was stirred for 30 min. The precipitate was discarded after centrifugation. To the supernatant, another 1 g of solid ammonium sulfate was added and the mixture was stirred for 30 min. After centrifugation, the precipitate was dissolved in 4.0 ml of the buffer and dialyzed against buffer overnight.

8) *Sephadex G-150 column chromatography* The dialysate (0.65 mg protein, 4.9 ml) after the 3rd ammonium sulfate step was applied to a Sephadex G-150 column (1 x 120 cm) equilibrated with buffer. Enzyme was eluted (1.5 ml/tube/ 6 min) with the same

buffer, the active fractions (Fr. No. 23-33) were pooled and concentrated to 3.0 ml with Amicon UM-10 membrane. This enzyme solution was used throughout this study.

Polyacrylamide gels Disk gel electrophoresis of native enzyme was carried out as described in section 1-2 of Chapter I.

Gel filtration Molecular weight determination was carried out with a column of Sephadex G-150 as described in section 1-2 of Chapter I.

RESULTS

Purification of γ -glutamylcysteine synthetase

As summarized in Table 1, γ -glutamylcysteine synthetase was purified about 2,200 fold from the extracts with the recovery of 8.3 % of the initial activity. The purified enzyme preparation showed a single band after polyacrylamide gel electrophoresis (Fig. 1). The molecular weight of this enzyme was determined to be 6×10^4 by gel filtration on Sephadex G-150 (Fig. 2). The polyacrylamide gel electrophoresis in the presence of sodium do-

Table 1. Summary of purification of γ -glutamylcysteine synthetase in *Escherichia coli* B

Step	Preparation	Protein (mg)	Specific activity (μ mole/h/mg protein)	Total activity (μ mole/h)	Yield (%)	Fold
1.	Crude extract	8420	0.171	1440	100	1.0
2.	1st ammonium sulfate (40%-70%)	3140	0.425	1340	92.7	2.5
3.	2nd ammonium sulfate (45%-70%)	774	1.35	1050	72.6	7.9
4.	DEAE-Cellulose	91.3	10.2	931	64.7	59
5.	Heat treatment (45 °C, 30 min)	7.72	83.2	642	44.6	486
6.	Hydroxyl apatite	2.41	197	475	33.0	1152
7.	3rd ammonium sulfate (45%-70%)	0.65	341	222	15.4	1994
8.	Sephadex G-150	0.32	374	120	8.33	2187

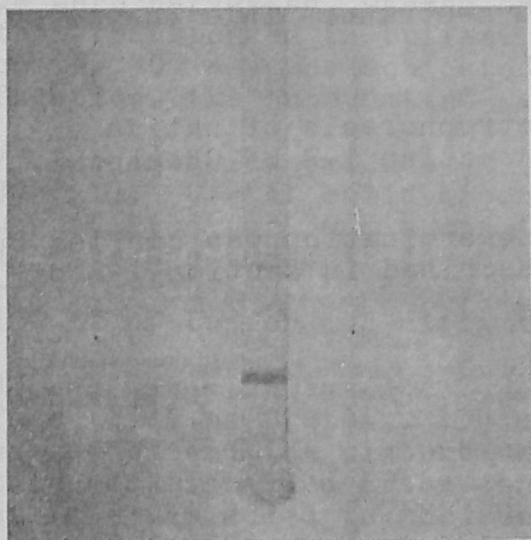


Fig. 1. SDS disk gel electrophoresis of γ -glutamylcysteine synthetase

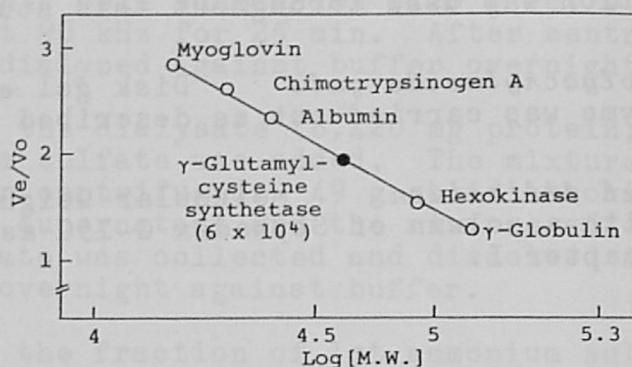


Fig. 2. Determination of molecular weight of γ -glutamylcysteine synthetase. Molecular weight of the enzyme was determined by gel filtration on Sephadex G-150. V_o and V_e are void volume of column and elution volume of proteins, respectively.

desyl sulfate failed to separate the enzyme into more than one component.

Enzymatic properties of γ -glutamylcysteine synthetase

1) *pH optimum* The optimal pH for activity was around 6.5 in Tris-HCl buffer (Fig. 3). A fifty % decrease in activity was observed when the buffer was substituted by potassium or sodium phosphate buffer (data are not shown).

2) *Effect of temperature* The optimal temperature for enzyme activity was 45°C (data not shown). Fig. 4 shows the temperature stability of this enzyme. Fifty % of initial activity was lost after treatment at 55°C for 35 min regardless of the presence or absence of Mg^{++} .

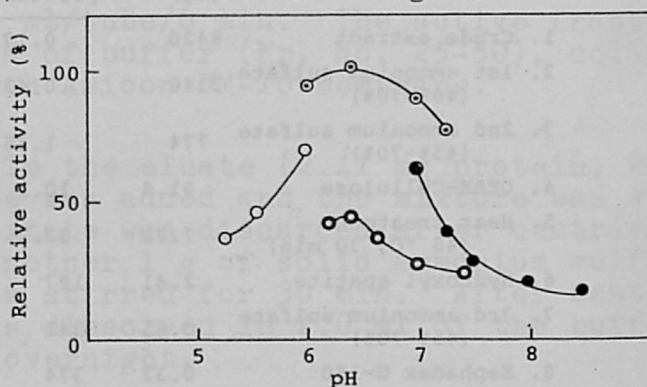


Fig. 3. Effect of pH on γ -glutamylcysteine synthetase activity. Buffers used were: (o), sodium acetate; (●), Tris-HCl; (⊙), triethanolamine-HCl and (⊗) potassium-phosphate. All buffers were used at the concentration of 0.1 M.

3) Metal ion requirement

This enzyme required divalent metal ions, especially Mg^{2+} , for activity (Table 2). The optimal concentration of Mg^{2+} was dependent on the ATP concentration (Fig. 5). The maximum activity was attained at the ratio of $ATP/Mg^{2+}=1/2$. Mn^{2+} , Ca^{2+} , and Zn^{2+} also could substitute for Mg^{2+} , though the enzyme activity was considerably decreased. Potassium ion, which is a activator of glutathione synthetase⁸²⁾ was not required. Sulfhydryl agents also inhibited this enzyme activity.

4) Nucleotides requirement

In addition to ATP, GTP and UTP were effective as energy sources for reaction. No significant activity was observed with CTP or ITP (Table 3).

5) Effect of inhibitors

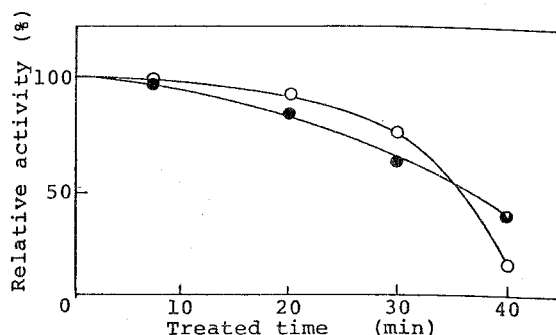


Fig. 4. Thermal stability of γ -glutamyl-cysteine synthetase. The enzyme solution was heated at 55 °C with (●) or without (○) 5 mM $MgCl_2$. At the prescribed time, aliquots of enzyme solution were taken, rapidly cooled and then used for the reaction as described in Materials and Methods.

Table 2. Effect of various compounds on γ -glutamyl-cysteine synthetase activity

Addition	Concn.	Relative activity(%)
None		10.2
$MgSO_4$	20 mM	100
$CaCl_4$	20 mM	24.8
$MnSO_4$	20 mM	25.6
$ZnSO_4$	20 mM	14.9
KCl	100 mM	98.2
Iodoacetamide*	5 mM	87.4
p-Chloromercury-benzoate*	1 mM	0
$HgCl_2$ *	2 mM	0

*) Preincubated with enzyme for 10 min at 37 °C

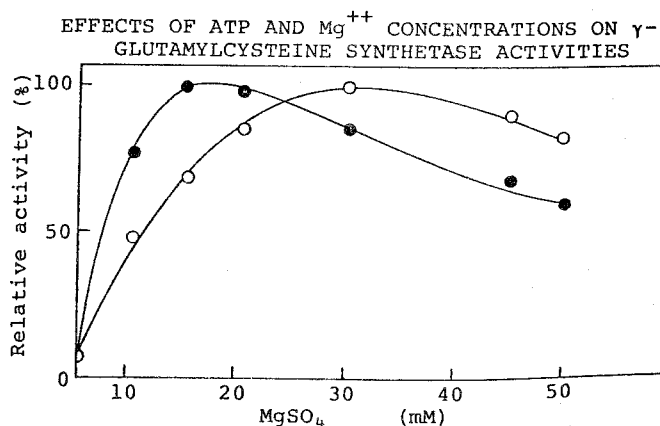


Fig. 5. Effect of $MgSO_4$ concentration on γ -glutamyl-cysteine synthetase activity. The reaction was carried out under the various concentration of $MgSO_4$ in the presence of 7.5 mM ATP (●) or 15 mM ATP (○).

Table 4 shows the effect of various inhibitors on γ -glutamylcysteine synthetase activity. This enzyme was inhibited by various agents such as cysteamine and cystamine. Inhibition by ADP was also observed, but its effect was lower in comparison with

the other inhibitors, though the data are not shown here. On the other hand, α -alkyl analogues of methionine, which are known as inhibitors of γ -glutamylcysteine synthetase of mammalian tissues^{82,84}), showed no inhibitory effect on the *E. coli* B enzyme.

6) *Effect of glutathione* The effect of reduced and oxidized glutathione on γ -glutamylcysteine synthetase activity was investigated (Fig. 6). The enzyme was specifically inhibited by reduced glutathione. Oxidized glutathione showed no inhibitory effect.

7) *Effect of phosphorus* Figure 7 shows the effect of potassium phosphate buffer and pyrophosphate. γ -Glutamylcys-

Table 3. Nucleotides requirement

Addition (15 mM)	Relative activity(%)
ATP	100
GTP	65.1
UTP	14.6
CTP	0
ITP	0

Reaction was carried out as described in Materials and Methods except for various nucleotides were used.

Table 4. Effect of various compounds on γ -glutamylcysteine synthetase activity

Addition (mM)	Relative activity(%)
None	100
L-Threonine thiohydantoin (5)	98.4
S-Allyl-DL-methionine (5)	97.3
α -Methyl-DL-methionine (5)	96.2
S-2-Aminoethyl cysteine (5)	94.6
L-Methionine-DL-sulfoxide (5)	97.3
L-Methionine sulfone (5)	99.1
DL-Methionine hydroxamate (5)	101
2-Aminoethyl mercaptan (10)	22.5
2,2'-Dithiobisethylamine (10)	8.21
N-Ethylmaleimide (5)	3.16

teine synthetase was severely inhibited by pyrophosphate. Potassium phosphate buffer also inhibited the enzyme activity, though the effect was less than that of pyrophosphate.

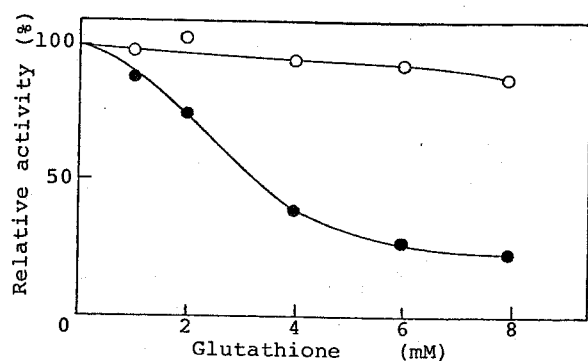


Fig. 6. Effect of glutathione on γ -glutamylcysteine synthetase activity. Reaction was carried out in the presence of reduced (●) or oxidized (○) glutathione at the concentrations indicated.

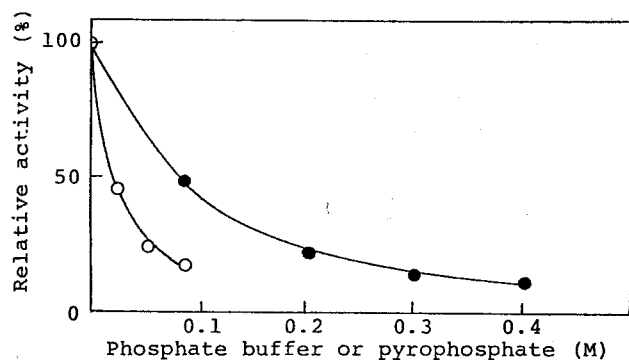


Fig. 7. Effects of pyrophosphate and phosphate buffer on γ -glutamylcysteine synthetase activity. Reaction was carried out as described in Materials and Methods except for the addition of pyrophosphate (○) or potassium phosphate buffer (pH 7.5) (●).

8) Effect of substrate concentration on γ -glutamylcysteine synthetase activity

The effect of substrate concentration on reaction rates was examined. The K_m values for L-glutamate, L-cysteine and ATP were calculated to be 1.2, 0.77 and 0.15 mM, respectively (Fig. 8).

DISCUSSION

γ -Glutamylcysteine synthetase was purified from *E. coli B*. This enzyme consisted of a single polypeptide chain having a molecular weight of 6×10^4 .

Analogous to the enzymes of various mammalian tissues, γ -glutamylcysteine synthetase of *E. coli B* required divalent metal ions for activity, and was inhibited by sulfhydryl reagents. The enzyme from *E. coli B* was also inhibited by iodoacetamide and ethylmaleimide. The effect of these inhibitors on the enzyme indicated that it was a sulfhydryl containing enzyme.

However, contrary to our expectation, only a slight inhibition of enzyme activity by α -alkyl analogues of methionine was observed, though the activities of enzyme from mammalian tissues were inhibited by these analogues^{82,84}). When the mammalian en-

zyme was incubated with analogues in the presence of ATP and Mg^{2+} , these analogues were phosphorylated, and bound tightly to the active site of the enzyme and inhibited enzyme activity⁸⁴). The insensitivity of the

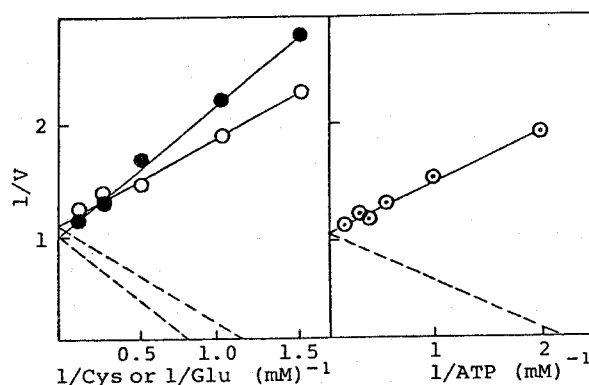


Fig. 8. Lineweaver-Burk⁵² plot for γ -glutamylcysteine synthetase reaction. Reaction was carried out as described in Materials and Methods except for the concentrations of substrates. Velocities are in arbitrary units. Symbols: (●), L-glutamate; (○), L-cysteine; (○), ATP

E. coli B enzyme to these analogues suggested that the reaction mechanism of the *E. coli B* enzyme is different from that of the enzyme from mammalian sources. Furthermore, the inhibition of the *E. coli B* enzyme by phosphorus (Fig. 7) has not been shown in the case of mammalian enzymes. Detailed studies on bacterial and mammalian enzymes are now required for an understanding of the differences between the enzymes.

Reduced glutathione, a end product of the glutathione synthetic pathway, also feedback inhibited the γ -glutamylcysteine synthetase, though oxidized glutathione did not. A similar result was also demonstrated by Apontoweil et al.⁸⁵). It is likely that the inhibition by reduced glutathione is of physiological significance. In fact, the concentration of 2.5 mM reduced glutathione needed to produce 50 % inhibition (Fig. 6) was comparable with the intracellular level measured in stationary phase cells (about 3.0 μ mole/ml packed cell). The repression of enzyme formation by glutathione might also occur, but our preliminary experiments excluded this possibility (data are not shown here). Therefore, as suggested by Apontoweil et al.⁸⁵), the glutathione synthetic pathway is controlled only by the

feedback inhibition by reduced glutathione.

From the results presented here, the release of feedback inhibition is indispensable for the production of glutathione by *E. coli B* cells. The simple structure and low molecular weight of the enzyme, γ -glutamylcysteine synthetase, encouraged me to (self)clone the gene responsible for this enzyme activity. These studies are described in the following section.

SUMMARY

γ -Glutamylcysteine synthetase was purified from *Escherichia coli B*. This enzyme consisted of single polypeptide chain having a molecular weight of 6×10^4 and required divalent cations for activity. The optimal pH and temperature for reaction were 6.5 and 45 °C, respectively. The K_m values for L-glutamate, L-cysteine and ATP were 1.2, 0.77 and 0.15 mM, respectively. GTP and UTP were also utilized in addition to ATP. This enzyme was inhibited by inorganic phosphate or pyrophosphate. Unlike the enzymes from mammalian tissues, the enzyme of *E. coli B* was not inhibited by α -alkyl analogues of methionine. The enzyme of *E. coli B* was severely inhibited by various sulfhydryl agents. The enzyme was also feedback inhibited by reduced glutathione, but not oxidized glutathione.

Section 4. Isolation of *Escherichia coli* B mutants deficient in glutathione biosynthesis and removal of feedback inhibition by glutathione

INTRODUCTION

As shown in previous section, the first enzyme in glutathione biosynthetic pathway was feedback inhibited by reduced glutathione. This inhibition was thought to be a barrier for the production of large amount of glutathione. So, the methods were sought to remove this inhibition. For this purpose, I employed a method to isolate the revertants of γ -glutamylcysteine synthetase deficient mutant, since it is empirically known that in some revertants of the mutants deficient in certain enzyme activity, the enzyme is desensitized from the feedback inhibition by end products.

The aim of the present investigation is to describe the easy method for isolating glutathione biosynthesis deficient mutants of *E. coli* B and also to obtain a strain having γ -glutamylcysteine synthetase desensitized from feedback inhibition by reduced glutathione.

METHODS AND MATERIALS

Chemicals Methylglyoxal (MG) and 8-hydroxyquinoline (8-HQ) were purchased from Sigma Chemical St. Louis. MO. γ -glutamylcysteine was prepared by the method of Strumeyer et al.⁸⁹.

Strains used M7, a cysteine auxotroph, and MGr or M910, both are MG-resistant strains, were used.

Isolation of glutathione biosynthesis deficient mutants MG(1.4 mM)-resistant mutant (MGr) was mutated with NTG by the method of Adelberg et al.⁹¹. The conditions of mutation were the same as described in section 1 of this Chapter. Mutated cells were spread on agar Davis-Mingioli minimal medium¹⁰⁰ (DM-medium) containing 1.4 mM MG and 10^7 cells/ml cysteine auxo-

troph (M7). After 2 days incubation at 37 °C, the colonies forming no halos were selected as mutants deficient in glutathione biosynthesis.

Isolation of a mutant having γ -glutamylcysteine synthetase desensitized from feedback inhibition by reduced glutathione

The mutant cells deficient in γ -glutamylcysteine synthetase activity were mutated with NTG by the method of Adelberg et al.⁹¹, and spread on the agr DM-medium containing 10 μ g/ml 8-HQ. After 2 days incubation at 37 °C, large colonies appeared were selected as revertants and tested for their ability to form γ -glutamylcysteine from L-glutamate and L-cysteine in the presence of reduced glutathione at the various concentrations.

Enzyme assay γ -Glutamylcysteine synthetase activity was determined by the method of Jackson⁸¹. Glutathione synthetase activity was determined by the method of Mooz and Meister⁸².

RESULTS AND DISCUSSION

Isolation of glutathione biosynthesis deficient mutants

As shown in Figure 1A, *E. coli* B 355 excreted little glutathione into the growth medium, and the growth of 355 was completely arrested by methylglyoxal (MG), a toxic metabolite for *E. coli* B, added to the medium at millimolar level. Contrary to strain 355, the MG-resistant mutant (MGr) was able not only to grow in the presence of higher concentration of MG, but also excreted large

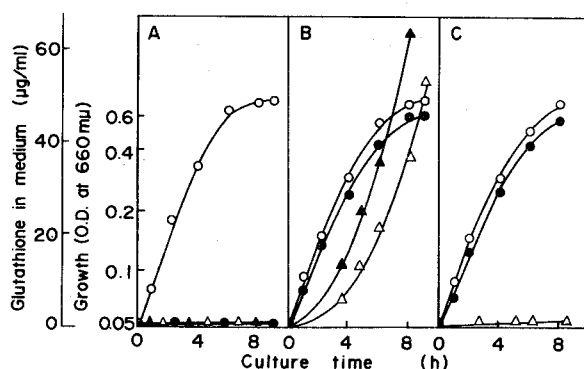


FIG. 1. Growth and Excretion of Glutathione by 355 (A), MGr (B) and by M-7 (C).

All the strains were grown on the medium described in Table I with or without the additions.

A and B: Growth in the absence (O) or presence (●) of 0.5 mM MG and excretion of glutathione in the absence (Δ) or presence (▲) of 0.5 mM MG.

C: Growth in the presence of 0.5 mM L-cysteine (O) or 0.1 mM glutathione (●) and excretion of glutathione in the presence of 0.5 mM L-cysteine (Δ).

amount of glutathione into the growth medium (Fig. 1B). This excretion of glutathione by MGr was further enhanced in the presence of MG (Fig. 1B). On the other hand, M7, a cysteine auxotroph, grew in medium containing cysteine or glutathi-

one (Fig. 1C), indicating that M7 can use the cysteine in glutathione. Therefore, by utilizing the properties of these two strains, MGr and M7, the glutathione synthesis deficient mutants will be isolated, since such mutants can not synthesize glutathione requiring for the growth of M7 embedded in the plates. So, MGr was mutated with NTG and spread on agar plates of minimal medium containing 10^7 cells/ml of M7. Plates were incubated and colonies forming no halos were isolated as glutathione biosynthesis deficient mutants.

Of about 50 colonies appearing on one plate, two or three colonies did not form halos, though the others formed. The properties of six colonies obtained from three sheets of plates were investigated (Table 1). Of the 7 colonies tested, two (C912 & C915) were deficient in γ -glutamylcysteine synthetase activity, and one (C1001) in glutathione synthetase activity. The other four (C900, C964, C1065 and C1198) synthesized glutathione at the same rates as the parent strain (MGr & M910).

Desensitization of γ -glutamylcysteine synthetase from feedback inhibition by reduced glutathione

Table 1. Activities of γ -glutamylcysteine and glutathione synthetase in mutant and parental strains

Mutant	Parent	Activity (μ mole/mg-protein/h)	
		γ -Glutamylcysteine synthetase	Glutathione synthetase
C900	M910	0.356	0.433
C912	M910	0	0.464
C915	M910	0.021	0.422
C964	M910	0.343	0.399
C1001	M910	0.342	0.003
C1065	M910	0.319	0.422
C1198	M910	0.354	0.463
RC912	C912	0.314	0.423
RC964	C912	0.289	0.399
-----		-----	
355	(wild)	0.149	0.192
M910	355	0.322	0.421

Reaction was carried out as described in Materials and Methods.

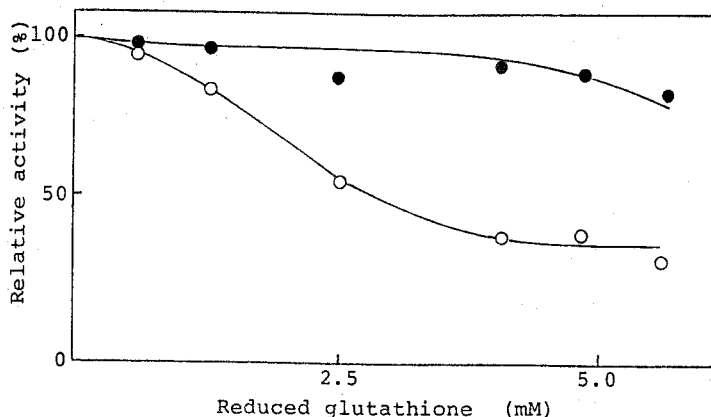


Fig. 2. Desensitization of γ -glutamylcysteine synthetase from feedback inhibition by reduced glutathione. Reaction was carried out in the presence of various concentrations of reduced glutathione. Activity in the absence of glutathione was taken as 100 %. (o), M910; (•), RC912

As the mutant deficient in γ -glutamylcysteine synthetase activity was obtained, the desensitization of this enzyme from feedback inhibition was investigated.

As described in Materials and Methods, several

revertants of C912 were isolated and γ -glutamylcysteine synthetase activities were determined in the presence of 3.0 mM reduced glutathione. Of the twenty colonies tested, one (RC912) showed higher activity even in the presence of reduced glutathione, though the enzyme activities in other revertants and MG-resistant strain (M910) severely inhibited by reduced glutathione (Fig. 2)

Thus, by using MG-resistant mutant in combination with M7, a cysteine auxotroph, the glutathione biosynthesis deficient mutants were easily isolated. Other method for isolating such mutants were described by Apontoweil et al.⁸⁵⁾ and by Fuchs et al.⁹⁵⁾, respectively. However, the utilization of the colony-colour technique developed by Apontoweil et al. is limited to the bacterial strains possessing an extremely low concentration of intracellular thiol compounds other than glutathione, and the isolation of mutants by this method is somewhat incidental as described by Apontoweil et al.. On the other hand, the method utilized by Fuchs et al. is logically consistent, but the pro-

cess for the construction of mutants is more time consuming than my technique presented here. The mutant obtained by my method was further utilized for the isolation of the strain having γ -glutamylcysteine synthetase desensitized from feedback inhibition by reduced glutathione. In fact, the γ -glutamylcysteine synthetase in revertant strain (RC912) derived from C912 was insensitive to the feedback inhibition by reduced glutathione (Fig. 2). This result may be a advantageous for the production of glutathione.

SUMMARY

By using a methylglyoxal-resistant mutant in combination with cysteine auxotroph, the glutathione biosynthesis deficient mutants were easily isolated. The mutants obtained were deficient in γ -glutamylcysteine synthetase activity (C912, C915) or glutathione synthetase activity (C1001). By determining the γ -glutamylcysteine synthetase activity in revertants of C912, the mutant (RC912) having γ -glutamylcysteine synthetase desensitized from the feedback inhibition by reduced glutathione was obtained.

Section 5. Some properties of glutathione biosynthesis deficient mutants of *Escherichia coli* B

INTRODUCTION

At present, there is almost lack of information concerning the role of glutathione in the functioning of the microbial cells, except the suggestions that this tripeptide is important in cell division⁹³⁾ and also in the detoxification of unfavorable compounds for growth⁸⁵⁾. However, more recently the importance of glutathione in the cells has been questioned by the finding that many gram positive bacteria⁹⁴⁾ and several mutants of *E. coli*^{85,95)} contain no detectable glutathione.

For the elucidation of the function of glutathione, I have been studying the metabolism of this tripeptide in *E. coli* B. One way of studying the metabolic role of glutathione is to observe the effect of intracellular oxidation of glutathione. For that purpose, azoester and diamide were introduced by Kosower et al.⁹⁶⁾, and t-butylhydroperoxide and cumene hydroperoxide by Srivastva et al.⁹⁸⁾. These agents were proven to be useful for the specific oxidation of intracellular glutathione without damaging the cell viability. However, the interpretation of the results obtained from the oxidation of glutathione was very complicated, since these agents caused a temporarily high concentration of oxidized glutathione in cells. Therefore, in studying the functions of glutathione biosynthesis, the use of the mutant cells with a deficiency in glutathione biosynthesis is to be preferred to the use of cells treated with the chemical agents.

As shown in previous section, I developed an efficient

method for the isolation of such mutants of *E. coli* B. Using this method, I obtained mutants deficient in the activity of γ -glutamylcysteine synthetase or glutathione synthetase.

This section deals with the properties of the mutants obtained and the physiological functions of glutathione in *E. coli* B.

METHODS AND MATERIALS

Chemicals Reduced and oxidized glutathione and acetyl phosphate were purchased from Sigma Chemical, St Louis, MO. γ -Glutamylcystein was prepared by the method of Strumeyer et al.⁹⁹.

Bacterial strains used

Bacterial strains used and their properties were listed in Table 1. In this Table, Mg^R indicates a gene for methylglyoxal (MG) resistance. The symbols *gsh I* and *gsh II* represent the gene responsible for the activities of γ -glutamylcysteine synthetase and glutathione synthetase, respectively.

Culture conditions and preparation of cell extracts

All the strains were cultivated in Davis-Mingioli minimal medium¹⁰⁰ with or without nutritional supplements or chemical agents. Glucose was separately autoclaved.

Level of glutathione and total thiol in cells Cells growing exponentially in Davis-Mingioli minimal medium were harvested and washed once with 0.85 % NaCl. Ethanol (3.0 ml, 80%) was added to 0.5 g wet weight cells and the thiol compounds were extracted from the cells by boiling for 5 min. The clear supernatants obtained after centrifugation were used for the determination of glutathione and total thiol compounds. Glutathione was determined by the method of Tietze⁴⁹. Total thiol compounds were determined by titration with Ellmans reagent¹⁰¹. All results were expressed as μ mole of glutathione or thiol compounds per g wet weight cells.

Determination of minimum inhibitory concentration of various agents

The gradient plate technique¹⁰² was used for the determination of minimum inhibitory concentration of various chemical agents. The method employed a press-bottom Petri plate (10 cm in diameter) with two layers of agar. The lower layer consisted of 10 ml of Davis-Mingioli minimal medium and was allowed to harden

Table 1. Strains used

Strain	Parent	Relevant properties
355	-	Wild-type
M71	355	<i>Cys</i> ⁻
M910	M71	Mg^R
C912	M910	Mg^R , <i>gsh I</i>
C915	M910	Mg^R , <i>gsh I</i>
RC912	C912	Mg^R
C1001	M910	Mg^R , <i>gsh II</i>

with the plate slanted to cover the entire bottom. After placing the dish in the horizontal position, another 10 ml of Davis-Mingioli minimal medium containing appropriate concentrations (C_0 $\mu\text{g/ml}$) of the chemical agents was added. The plates were incubated at 37 °C for 24 h after streaking with cells. The length of the growing streak (L cm) was measured and the minimum inhibitory concentrations (C $\mu\text{g/ml}$) was calculated based on the equation: $C = LC_0/10$.

Survival curves The cells growing in exponential phase on Davis-Mingioli minimal medium were harvested and resuspended in 3.0 ml of the same medium containing the chemicals to be tested at a density of 5×10^8 cells/ml. The suspension was incubated at 37 °C with shaking. At the various times, samples were taken, diluted, and spread on L-broth agar plates. The composition of L-broth was described in section 3 of this Chapter. The plates were incubated at 37 °C to determine the number of surviving cells.

RESULTS

Glutathione and total thiol compound concentration in cells

The amount of intracellular glutathione and total thiol compounds in the mutant and parental strains were determined and the results are presented in Table 2. In the parental strains (355 and M910), glutathione represents about 35-40 % of total thiol compounds. As suggested previously in section 2,

methylglyoxal resis-

tance resulted in an

increase in the levels

of glutathione and to-

tal thiol compounds.

On the other hand, in

the mutant strains (

(C912 and C1001), sig-

nificant level of glu-

tathione were not detected. Increased levels of glutathione and total thiol compounds were found in RC912, a revertant of C912.

Table 2. Glutathione and total thiol content of mutant and parental strains

Strain	Sensitivity to methylglyoxal	Total thiol ($\mu\text{mole/g-cells}$)	Glutathione ($\mu\text{mole/g-cells}$)
355	sensitive	8.2	2.9
M910	resistant	9.6	3.8
C912	resistant	3.9	0.04
C1001	resistant	4.9	0.02
RC912	resistant	10.7	4.7

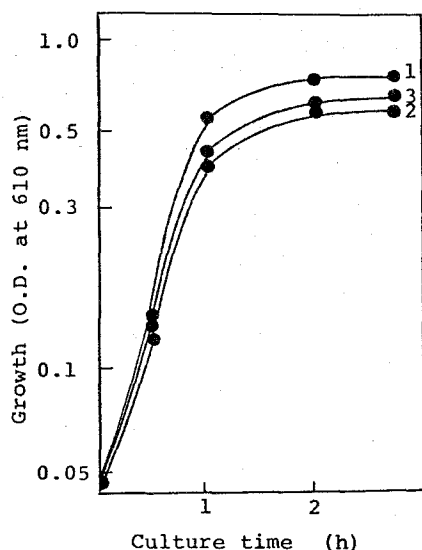


Fig. 1. Growth of mutant and parental strains on minimal medium. Overnight culture in Davis-Mingioli minimal medium was harvested and washed once with 0.85 % NaCl. Samples of cells were resuspended in 3.0 ml of the same minimal medium with or without 10^{-4} M glutathione, and incubated at 37 °C with shaking. Curve 1: M910; Curve 2: C912; Curve 3: Cl001.

Growth on minimal medium

Figure 1 shows the growth of the mutant and parental strains on Davis-Mingioli minimal medium. The maximum levels of growth of the mutant strains were somewhat lower than that of the parental strain. The addition of glutathione in the medium of C912 did not improve the growth.

Susceptibility to inhibitors

The susceptibility of the mutant strains to various chemical agents was investigated, since glutathione is thought to be responsible for the detoxification of the compounds unfavorable to growth. The kinds of chemicals tested were typical sulfhydryl reagents (HgCl_2 Iodoacetoamide), a metal chelating reagent (8-Hydroxyquinoline), an antibiotic (Chloramphenicol) and others (mostly were inhibitors of γ -glutamyltranspeptidase). As can be seen in Table 3, the γ -glutamylcysteine synthetase deficient strain (C912) was highly susceptible to various inhibitors, but not to except chloramphenicol, though glutathione synthetase deficient strain (Cl001) was somewhat resistant to these chemicals. The specific interactions of these chemicals with glutathione were not elucidated in this study.

Figure 2

Table 3. Minimal inhibitory concentration of various agents

Chemicals	Minimal inhibitory concentration ($\mu\text{g/ml}$)			
	355	M910	C912	C1001
HgCl ₂	4.0	5.0	0.1	0.4
Iodoacetamide	75	92	20	40
Tetramethylthiuram- disulfide (TMTD) *	91	100	5.5	10
8-Hydroxyquinoline (8-HQ)	16	20	4.1	10
Propyl 4-hydroxybenzoate	98	120	40	75
Methylglyoxal	28	40	5.5	7.7
Chloramphenicol	3.5	4.0	8.0	8.5
Dibromophenol	120	140	77	89
m-Cresol	1000	1000	900	1000
Tetrabromo-m-cresol	500	550	50	120
Thymol	250	300	150	200
Phenol-disulfonate- tetrabromo	95	130	55	75
Chloromethylketone	250	250	80	110

* $(\text{CH}_3)_2\text{NCSS}_2\text{CSN}(\text{CH}_3)_2$

Minimal inhibitory concentration was determined as described in Materials and Methods.

shows the change in viability of cells exposed to tetramethylthiuram disulfide (TMTD) and 8-hydroxyquinoline (8-HQ). A marked decrease in viability was found in the γ -glutamyl-

cysteine synthetase deficient strain. The addition of glutathione into the mixture with TMTD or 8-HQ protected the cells of C912.

Susceptibility to oxygen

Figure 3 shows the growth of mutant and parental strains on Davis-Mingioli minimal medium containing 10 $\mu\text{g/ml}$ of TMTD. Marked inhibition of growth of M910 at this concentration of TMTD was not observed irrespective of the volume of medium. The inhibition of the growth of the γ -glutamylcysteine synthetase deficient strain (C912) was increased when the volume of the medium was decreased. The effect of culture volume on the growth was not so severe for the glutathione synthetase deficient mutant (C1001).

DISCUSSION

Mutant of *E. coli* B deficient in glutathione biosynthesis were easily isolated by a previously described method. An alter-

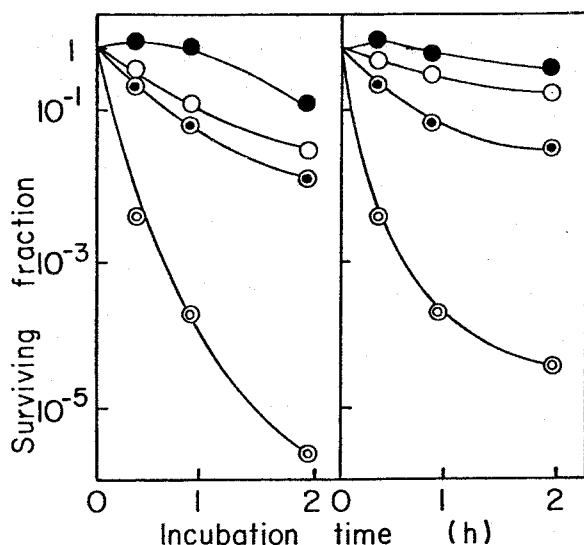


FIG. 2. Decrease in living cells during incubation in the presence of TMTD (left) and 8-HQ (right).

The cells logarithmic growth in Davis-Mingioli minimal medium were harvested, washed once with 0.85 % saline solution and resuspended in 3.0 ml of the same medium containing 50 µg/ml TMTD or 40 µg/ml 8-HQ. C912 was also incubated in the presence of TMTD and 10^{-4} M glutathione. The incubation was carried out at 37 °C with shaking. The number of viable cells after treatment was determined by counting the colonies that appeared on the nutrient agar plate as described in Materials and Methods. (●), M910; (○), C1001; (◐), C912; (◑), C912 + 10^{-4} M glutathione.

native method for the isolation of such mutants, a colony colour technique, has also been reported by Apontoweil et al. However, the application of their method is limited to bacterial strains

containing extremely low concentration of intracellular thiol compounds other than glutathione. Cells containing larger quantities of thiol compounds will still produce a red colour even in the absence of glutathione. This is the case with *E. coli* B, thus the isolation of glutathione biosynthesis deficient mutants is difficult by their method. My method, using a cysteine auxotroph in combination with a methylglyoxal-resistant mutant is very simple and easy, and may be applied to all bacterial strains regardless of the quantities of intracellular thiol compounds. Two kinds of mutants were isolated. Enzymatic analysis showed that one kind lacked γ -glutamylcysteine synthetase activity (C912 & C915) and the other glutathione synthetase activity (C1001).

The properties of the glutathione biosynthesis deficient *E. coli* B mutants were investigated. Glutathione deficiency did

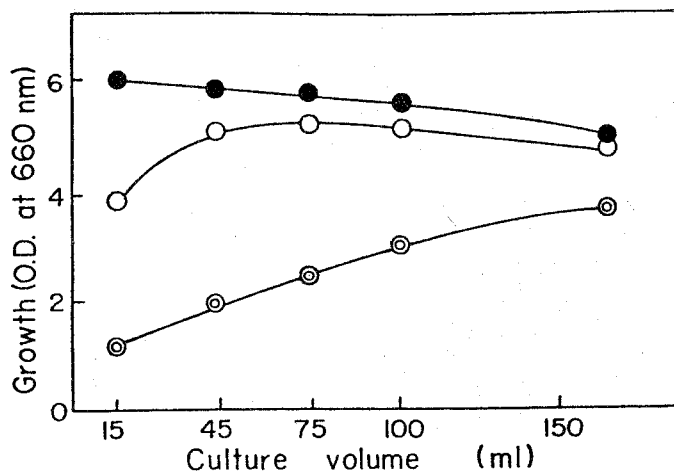


FIG. 3. Effect of culture volume on the growth of mutant and parental strains.

Cells were grown on various volume of Davis-Mingioli minimal medium containing 10 μ g/ml TMTD. Incubation was carried out at 37 °C with shaking for 32 h using 5 dl Sakaguchi flasks. (●), M910; (○), C912; (⊙), C1001

not significantly affect growth on minimal medium (Fig. 1). As originally suggested by Apontoweil et al.⁸⁵⁾ and by Fuchs et al.⁹⁵⁾, this indicates that glutathione is not specifically required for any process in the cells for their proliferation.

This was of considerable interest, since

glutathione represents approximately 40 % of the total thiol compounds in *E. coli* B cells (Table 3).

As can be seen in Figure 2 and Table 3, glutathione is a significant factor for the protection of cells exposed to toxic compounds. These results are the same as those described by Apontoweil et al.. The mutant deficient in γ -glutamylcysteine synthetase activity was more susceptible to various chemical agents than the parental or the mutant lacking in glutathione synthetase activity. It seems likely that γ -glutamylcysteine, a product of the γ -glutamylcysteine synthetase reaction, can substitute for glutathione in some, but not all instances. This result indicated that the mutation was brought about only in the region of *gsh I*, but not in *gsh II*, though NTG treatment usually induces multiple mutations⁹¹⁾. Kosower and Kosower⁹⁷⁾ showed that lysis occurs when a chemical challenge is given in the absence of glutathione and they termed this phenomenon the "glutathione loss

catastrophe". The same phenomenon was recognized by many other investigators, though the chemical interactions of glutathione with these substances remain obscure. The glutathione biosynthesis deficient mutants were also susceptible to the amount of aeration unlike the parental strains (Fig. 3). A high susceptibility to lysozyme treatment was also found for the glutathione biosynthesis deficient mutants (data not shown). Thus glutathione may play an important role in the maintenance of the rigidity of cell walls.

SUMMARY

Mutants of *Escherichia coli* B that contain essentially no detectable glutathione were isolated. These mutants contain a very low level of γ -glutamylcysteine synthetase or glutathione synthetase activity. No significant differences in growth on minimal medium were observed between the mutants and the parental strains. The mutants lacking γ -glutamylcysteine synthetase activity were more highly susceptible to toxic compounds than the parental or a glutathione synthetase deficient strain. The mutants lacking γ -glutamylcysteine synthetase activity were also found to be susceptible to oxygen.

Section 6. Self-cloning of a gene responsible for the biosynthesis of glutathione in *Escherichia coli* B

INTRODUCTION

I have been studying the production of glutathione in a bioreactor system containing an ATP regeneration process. As shown previously, this tripeptide was found to be continuously produced by a column packed with immobilized *E. coli* B cells or immobilized *Saccharomyces cerevisiae* cells, though the amount produced was relatively lower in comparison with the present organic synthesis or extraction from the yeast cells.

This low productivity of glutathione of my bioreactor systems was presumably due to the feedback inhibition of γ -glutamylcysteine synthetase (GSH-I), the first enzyme in the glutathione biosynthetic pathway, by reduced glutathione. To improve these systems, I investigated the cloning of a gene responsible for the activity of GSH-I, which was released from the feedback inhibition by reduced glutathione. As a result, I succeeded in the construction of *E. coli* B strains having high glutathione-synthesizing activity.

This section deals with these results.

MATERIALS AND METHODS

Chemicals Restriction endonucleases (Eco RI, Bam HI, Hind III, and Pst I) and T₄ DNA ligase were purchased from Takara Shuzo Co., Ltd., Tokyo, Japan. Tetramethylthiuram disulfide (TMTD) and other reagents used for the assay for γ -glutamylcysteine synthetase activity were purchased from Sigma Chemical Co. St. Louis, MO.

Strains used Strains used were listed in Table 1. All the mutant strains were derived from the *Escherichia coli* B (355). The *gsh I* indicates the gene coding for γ -glutamylcysteine synthetase. *Mg^R* indicates the methylglyoxal resistance. The *gsh I⁺*

indicates the functional gene after back mutation of *gsh I*⁻ of C912.

Table 1. Strains used

Strain	Parent	Properties
M910	355(wild)	<i>Mg</i> ^R
C912	M910	<i>Mg</i> ^R , <i>gsh I</i>
RC912	C912	<i>Mg</i> ^R , <i>gsh I</i> ⁺

DNA Chromosomal DNA of RC912 was purified according to the method of Saito and Miura¹⁰³. Plasmid DNA was purified by the method of Tanaka and Weisblum¹⁰⁴.

Digestion and ligation of DNA

Digestion of DNA was carried out with Eco RI in 0.1 M Tris-HCl (pH 7.5), 50 mM MgCl₂, with Bam HI in 50 mM Tris-HCl (pH 7.5), 0.2 mM EDTA and 5 mM MgCl₂, and with

Pst I or Hid III in 6 mM Tris-HCl (pH 7.5), 50 mM NaCl and 6 mM MgCl₂. Digestion was carried out at 37 °C in a volume of 0.1 ml. Ligation with T₄ DNA ligase was carried out by the method of Tanaka and Weisblum¹⁰⁴.

Electrophoresis in agarose gels Agarose gels (slab type, 1.5 %) were run in the same buffer as those described by Helling et al.¹⁰⁵.

Transformation and selection Transformation of *E. coli B* strains was carried out as described by Nagahari et al.¹⁰⁶. Selection of transformants was as follows. About 10⁵ cells after incubation with whole ligation mixture were spread on a plate of Davis-Mingioli minimal medium¹⁰⁰ supplemented with 90 µg/ml TMTD. Plates were incubated at 37 °C for 2 days and the colonies appeared were tested for their sensitivity to antibiotics. Antibiotics used for the selection were ampicillin 10 µg/ml and tetracycline 10 µg/ml in L-broth comprising of 1.0 % polypeptone, 0.5 % yeast extract, 0.5 % NaCl and 0.1 % glucose (pH 7.2). The colonies resistant to TMTD and ampicillin and/or tetracycline were further tested for their ability to produce red colour in the presence of nitroprusside by the method of Apontoweil et al.⁸⁵.

Assay for γ-glutamylcysteine synthetase (GSH-I) Strains of *E. coli B* with and without hybrid plasmid were grown to log phase at 37 °C in Davis-Mingioli minimal medium. Preparation of cell extracts and assay for GSH-I activity were carried out as described in section 2 of this Chapter.

Glutathione contents Intracellular glutathione level was determined by the method as previously described in section 2 of this Chapter.

Purification of GSH-I The purification of GSH-I from the strains of *E. coli B* with and without hybrid plasmid was carried out as previously described in section 3 of this Chapter.

RESULTS

Construction of hybrid plasmid

RC912 chromosomal DNA (1.2 μ g) was partially digested with appropriately diluted restriction endonucleases for 1.5 and 6 h. Vector plasmid pBR322 (1.5 μ g) was also digested completely with the same nucleases used for the digestion of chromosomal DNA. These two kinds of digestion products were ligated with T_4 DNA ligase. When the cells of C912 were transformed with the whole ligation mixture, several TMTD resistant colonies appeared, though the number of colonies on the plates were dependent on both the kind of restriction endonuclease used and digestion time (Table 2).

The TMTD resistant colonies were further tested for their sensitivity to antibiotics. The colonies obtained by digestion with Bam HI and Hind III were resistant to ampicillin and sensitive to tetracyclin, with Pst I, they were sensitive to ampicillin and resistant to tetracyclin, and with Eco RI, they were resistant to ampicillin (Data not shown).

Colony colour

The colonies resistant to TMTD and antibiotics used were

Table 2. Effect of digestion time and kind of endonuclease on the appearance of TMTD resistant colonies

Restriction endonuclease	Digestion time(h)	No of colonies appeared/ 10^9 cells	No of colour producing colonies
Hind III	1.5	1	0
	6.0	6	1 (H3)
Bam HI	1.5	0	0
	6.0	20	0
Eco RI	1.5	22	2 (E7, E11)
	6.0	0	0
Pst I	1.5	2	1 (P1)
	6.0	9	0

further tested for their ability to produce red colour in the presence of nitroprusside. Contrary to my expectation, the number of resistant colonies reacting with nitroprusside was only a few % (5-10 %) of the colonies obtained, and almost all colonies remained white-pink in colour. Number of colonies which

Table 3. Glutathione content and GSH-I activity in transformants

Strain	Glutathione content (μ mole/g-cells)	GSH-I activity (μ mole/mg-protein/h)
H3	3.16	1.08
E7	1.34	0.64
E11	1.54	0.43
P1	1.98	0.66

Strains used were listed in Table 2.

produced red colour were: 1 (designated H3) with Hind III, 0 with Bam HI, 2 (E7, E11) with Eco RI and 1 (P1) with Pst I (Table 2).

Glutathione contents and GSH-I activity of transformants

The glutathione contents and GSH-I activity of strains producing red colour were determined (Table 3). An accumulation of glutathione in cells and GSH-I activity were observed in all the transformants obtained. Among these strains, H3 showed the highest GSH-I activity.

Transformation of RC912 with hybrid plasmid pBR322-gsh I

The hybrid plasmid, designated pBR322-gsh I, carrying by H3 was isolated and used for the transformation of RC912. Transformants of RC912 were selected on the L-broth containing ampicillin (10 μ g/ml). Several ampicillin resistant colonies were isolated on the plates and these colonies were also sensitive to tetracyclin. The frequency of transformation of RC912 with pBR322-gsh I was about 2×10^4 / μ g plasmid DNA. One of the transformants was designated RC912/pBR322-gsh I.



Fig. 1. Agarose gel electrophoresis of pBR322-*gsh I* plasmid DNA digested with various restriction endonucleases. The Hind III generated fragments of λ cI857S7 phage DNA are used as molecular weight standards. The size of the fragments are (from top in Md) 14.63, 6.13, 4.05, 2.85, 1.45, 1.26 and 0.43¹⁰⁷. (1), λ cI857S7 digested with Hind III; (2), pBR322 digested with Hind III; (3), pBR322-*gsh I*; (4,5,6), pBR322-*gsh I* digested with (4) Hind III, (5) Bam HI, (6), Pst I.

Characterization of pBR322-gsh I

The hybrid plasmid pBR322-*gsh I* was purified from C912/pBR-322-*gsh I*. Figure 1 shows the electrophoretic pattern of this plasmid digested with various restriction endonucleases. The molecular weight of pBR322-*gsh I* was 6.2 Md, indicating that a 3.4 Md segment of chromosomal DNA of RC912 was inserted into the Hind III restriction site of pBR322. The pBR322-*gsh I* was susceptible to Bam HI, Pst I, Eco RI and Hind III. The susceptible sites of these nucleases were determined as shown in Figure 2, except for the sites for Eco RI. The sites for Eco RI were not determined, since this nuclease generated 5 fragments having a molecular weight of 2.3, 1.8, 1.1, 0.8 and 0.2 Md.

Properties of RC912/pBR322-gsh I

The properties of RC912/pBR322-*gsh I* were investigated and compared with those of parental strains (Table 4). The glutathione content and GSH-I activity of RC912/pBR322-*gsh I* increased about 7- and 12-fold, respectively, as compared with those of

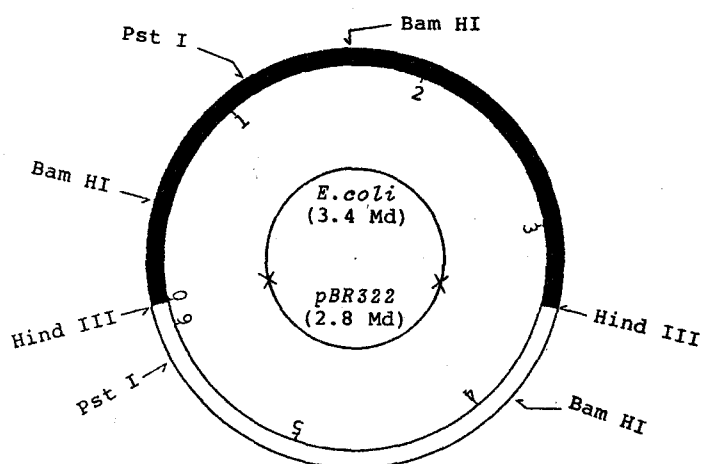


Fig. 2. Circular restriction map of pBR322-*gsh I*.

The restriction map is based on the relative size of the restriction fragments. The arrows indicate the points of restriction endonuclease attack. The numbers indicate the size of the fragments in megadaltons. X indicates the site where the Hind III ends of pBR322 were ligated to a fragment of *E. coli B* (RC912) chromosomal DNA.

Table 4. Construction of *Escherichia coli B* strains having high glutathione-synthesizing activities

Strain	Properties	GSH content ¹	GSH-I activity ²	Overall GSH-synthesizing activity ³
355	wild	0.71	0.13	0.13
M910	<i>mg</i> ^r	1.48	0.38	0.27
C912	<i>mg</i> ^r , <i>gsh I</i> ⁻	0	0	0
RC912	<i>mg</i> ^r , (<i>gsh I</i> ⁺)	2.45	0.43	0.31
RC912/pBR-322- <i>gsh I</i>	<i>mg</i> ^r , (<i>gsh I</i> ⁺)	4.81	1.76	1.26

1) mg/g(wet wt.)-cells

2) μ mole/mg-protein/h

3) μ mole/mg-protein/h

strain 355. 10-fold increase in overall glutathione synthesizing activity was also found in this transformant.

The GSH-I in RC912/pBR322-*gsh I* was isolated and enzymatic properties of this enzyme were investigated (Table 5). The molecular weight, optimal pH and temperature for reaction and specific activity of the transformant were the same as those of M910. The enzyme from RC912/pBR322-*gsh I* was not in-

hibited by reduced glutathione, though the enzyme from M910 did. The resistance of enzyme from RC912/pBR322-*gsh I* to various inhibitors was found to be higher than that of M910.

DISCUSSION

Table 5. Comparison of properties of γ -glutamylcysteine synthetases

Properties	Enzyme from	
	M910	RC912/pBR322- <i>gsh I</i>
M.W.	60,000	60,000
Optimal pH	6.5	7.0
Optimal temperature(°C)	40	40
Specific activity (μ mole/h/mg-protein)	374	386
Km (mM) for L-glutamate	1.20	0.92
L-cysteine	0.76	0.52
ATP	0.50	0.41
Inhibition ¹ by		
reduced glutathione (mM)	3.5	-
cysteamine (mM)	20	35
cystamine (mM)	3.0	11
pyrophosphate (mM)	25	30
phosphate buffer (M)	0.10	0.10

1) Concentration required for 50 % inhibition

The self-cloning of a gene responsible for the γ -glutamylcysteine synthetase (GSH-I) was investigated. Two factors influenced on the appearance of transformants carrying a hybrid plasmid pBR322-*gsh I*. One was the ef-

fect of digestion time and kind of restriction endonuclease used (Table 2). Two possibilities can be considered to explain this effect: (1) the differences in susceptibility of the gene (*gsh I*) to the endonuclease used. Longer digestion time may cause damage to *gsh I*. Restriction endonuclease having many site in *gsh I* also cleaved the gene and produced non-functional gene fragments, even when the digestion time was short, (2) the differences of transforming-activity of pBR322-*gsh I* prepared by various endonucleases. Harris-Warrich et al.¹⁰⁸⁾ indicated that the transforming-activity of Eco RI-digested chromosomal DNA depends not only on the molecular weight of the fragments, but also on the distance of the gene from a cleaved site. However, clear relationship between digestion time and the frequency of appearance of transformants was not observed in this study.

Second, almost all the TMTD-resistant colonies did not produce red colour, which is a characteristic colour of colonies containing sulfur compounds in large quantity. This result indicated that the susceptibility to TMTD was not always a specific

character of the glutathione biosynthesis deficient mutant (C912) and this susceptibility was neutralized by many factors other than glutathione, though such factors were not elucidated.

The enzymatic properties of GSH-I of RC912/pBR322-*gsh I* were investigated and compared with those of parental strain(M910) (Table 5). As expected, the enzyme was released from the feedback inhibition by reduced glutathione, and 12-fold increase in GSH-I activity was attained. These two results suggested that the strain RC912/pBR322-*gsh I* may be used for the industrial scale production of glutathione.

SUMMARY

Self-cloning of a gene (*gsh I*) responsible for the γ -glutamylcysteine synthetase (GSH-I) activity was studied to construct a *Escherichia coli* B strain having high glutathione-producing activity. For this purpose, two kinds of *E. coli* B mutants (C912, RC912) were used. C912 was a mutant deficient in GSH-I activity. RC912 was a revertant of C912, of which GSH-I was desensitized from the feedback inhibition by reduced glutathione. To clone a *gsh I*, the chromosomal DNA of RC912 and vector plasmid pBR322 were digested by various restriction endonucleases and then ligated with T₄ DNA ligase. The ligation mixture was incubated with C912, and the transformants were selected as TMTD- and antibiotics resistant colonies. Of about 20 resistant colonies, two or three produced red colour by reacting with nitroprusside and showed appreciably high GSH-I activities. The chimeric DNA, designated pBR322-*gsh I*, was isolated from the strain having the highest GSH-I activity and cloned to RC912. The structure and

molecular size of pBR322-*gsh I* were determined. The molecular weight of this plasmid was 6.2 megadalton and contained a 3.2 megadalton segment derived from the RC912 chromosomal DNA, including the *gsh I* gene. The GSH-I activity of RC912 cells containing pBR322-*gsh I* was 4-fold higher than that of RC912 cells.

CONCLUSION

In this study, the production of useful compounds using microbiological energy was investigated.

In Chapter I, the availability of energy in phosphate polymers for the production of G-6-P and NADP was studied. As a result, these two compounds were found to be efficiently produced from glucose or NAD using phosphate polymers, especially metaphosphate, as a donor of energy and phosphorus. In the course of this study, I obtained following two interesting results. These are: (1) the enzyme catalyzing the phosphorylation of NAD using metaphosphate was novel enzyme, different from the ATP-dependent NAD kinase. (2) The bacterial strains having G-6-P and NADP forming activities were mainly found in the two genera, *Micrococcus* and *Brevibacterium* species. This result may provide some suggestions for the classification and evolution of these microorganisms, since in ancient microorganisms, phosphate polymers may play an important role in energy metabolism usually inherent to ATP.

In Chapter II, the availability and feasibility of various ATP regeneration systems were investigated to construct highly efficient bioreactor for the production of glutathione. Among the ATP regeneration systems examined, glycolytic pathway in yeast cells was more promising on both the ATP regenerating activity and operational stability. Especially, the coupled production system of *S. cerevisiae* cells with *E. coli* B cells was most efficient than other reactor systems investigated. So, the biochemical and gene engineering studies were carried out to construct a *E. coli* B strains having high glutathione-producing activity.

First, to increase in glutathione-producing activity, I obtained methylglyoxal(MG)-resistant mutants and characterized their properties. Two fold increase in glutathione-producing activity was found in MG-resistant mutant. Another characteristic property of MG-resistant mutant was the excretion of glutathione out of cells into growth medium. This property of MG-resistant mutant greatly enhanced the isolation of glutathione-biosynthesis deficient mutants of *E. coli B*.

Two kinds of glutathione biosynthesis deficient mutants were isolated. One was deficient in γ -glutamylcysteine synthetase, the other in glutathione synthetase. These two kinds of mutants could grow on the minimal medium without any supplementation. However, the mutants deficient in glutathione biosynthesis were highly susceptible to chemical agents such as metal chelating agents, antibiotics and sulfhydryl agents. These results indicated that the glutathione protect the cells exposed to the unfavourable compounds for growth.

Next to this study, I investigated the properties of γ -glutamylcysteine synthetase, a first enzyme in glutathione biosynthesis, to elucidate the regulation mechanism in glutathione biosynthetic pathway in *E. coli B*. This enzyme consisted of single polypeptide chain having a molecular weight of 6×10^4 and was feedback inhibited by reduced glutathione, but not oxidized glutathione. Therefore, the construction of mutant strains desensitized from the feedback inhibition by reduced glutathione was thought to be indispensable for the production of glutathione in large quantity. Such strains were easily obtained in the revertants of the strains deficient in γ -glutamylcysteine synthetase.

The single structure of γ -glutamylcysteine synthetase and

the construction of desensitized strain from the feedback inhibition facilitated the cloning of a gene responsible for the γ -glutamylcysteine synthetase activity. As a result, I succeeded in the construction of a *E. coli B* strain having high glutathione synthesizing activity. The methods I developed here for the construction of *E. coli B* cells may be applicable to all the production of useful compounds other than glutathione. That is, this method I employed showed that the gene engineering technics is applicable not only to the production of proteins and peptides, but also to the large scale production of various microbial metabolites.

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